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Immunochemical Studies on a Galactosyl-Protein Conjugate †

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(*Received 25 February 1960*)

Antibodies formed in rabbits immunized with galactosido-β-D-azophenyl bovine serum albumin, after absorption with bovine serum albumin, were studied by quantitative hapten-inhibition techniques. It was shown (a) that the surface profile of the antibody is specific for the β-D-galactopyranosyl structure, (b) that the dimension of the combining site is complementary to the galactopyranosyl-phenylazo moiety and is approximately half that of the previously well-defined human antidextran site, (c) that the antibody or spectrum of antibodies in sera from two rabbits present an apparent micro-identity.

1. Introduction

The assumption has frequently been made that there is a similarity between induced enzyme and antibody formation. In a previous paper (Tanenbaum, Mage & Beiser, 1959), this hypothesis was strengthened by a comparison of the abilities of various substances to induce the formation of β-galactosidase with their capacities to combine with rabbit anti-β-D-galactoside antibody. During the course of these studies, the specificity of anti-β-D-galactosido-antibodies, first studied qualitatively by Avery & Goebel (1929), was reinvestigated using quantitative hapten-inhibition techniques. The results reported here pertain to the size of the combining site, the heterogeneity of antibodies, and extend the earlier work on the specificity of this antibody.

2. Materials and Methods

(a) *Haptens*

p-Nitrophenyl-β-D-galactoside and *p*-aminophenyl-β-D-galactoside were synthesized by a modification of the original synthesis (Avery & Goebel, 1929). Acetobromo-galactose was condensed with *p*-nitrophenol over K_2CO_3 in H_2O-acetone (Latham, May & Mosettig, 1950), and the resultant *p*-nitrophenyl-β-D-galactoside tetraacetate, m.p. 139 to 140°C, was deacetylated in methanol with ammonia to yield *p*-nitrophenyl-β-D-galactoside, m.p. 173 to 175°C. Catalytic reduction in ethanol at atmospheric pressure over PtO_2 and recrystallization afforded *p*-aminophenyl-β-D-galactoside, m.p. 153°C, $[\alpha]_D = -40{\cdot}3°$ ($c = 1{\cdot}0\%$ in absolute methanol).

Tyrosine-azophenyl-β-D-galactoside, histidine-azophenyl-β-D-galactoside and the other related azo dyes made from the *p*-aminophenyl sugar were prepared as impure substances, exemplified by the following: 0·1 m-mole tyrosine (18 mg) was dissolved in 8 ml. H_2O, treated with 0·1 m-mole NaOH and chilled. In a separate test tube, 27 mg *p*-aminophenyl-β-D-galactoside (0·1 m-mole) was dissolved in 2·0 ml. of 0·1 N-HCl (0·2 m-mole), chilled, and 6·9 mg $NaNO_2$ (0·1 m-mole) was added at once. After shaking in the cold for 5 min, the two solutions were mixed and kept at 0°C for 1 hr. The neutral reaction mixture was then lyophilized to give a completely water-soluble powder containing about 24% NaCl by weight. Comparison of the ultraviolet absorption spectra of these diazo compounds

† Supported by a Grant (E-1763) from the U.S. Public Health Service.

with published spectra (Tabachnick & Sobotka, 1959) for diazo derivatives of aromatic amino acids indicated that only the monoazo species were formed by this procedure. Paper chromatographic analyses of the diazo compounds using the butanol : acetic acid : H_2O (4 : 1 : 1) solvent system and the ninhydrin spray reaction indicated that only trace quantities (less than 5%) of the original aminophenyl glycoside and the uncoupled aromatic compound remained.

Other galactosides used in these experiments were obtained through the kind courtesies of Drs. M. Cohn, H. O. Halvorson, H. S. Isbell, N. K. Richtmyer, S. Spiegelman and M. L. Wolfrom.

(b) *Antigen*

The aminophenyl glycoside was coupled to bovine serum albumin, Armour fraction V (BSA), using the procedure outlined by Westphal & Feier (1956). To 1·0 g BSA dissolved in 50 ml. of 0·04 N-NaOH and then chilled was added a cold solution of 165 mg *p*-aminophenyl-β-D-galactoside in 10·2 ml. of 0·109 N-HCl containing 40 mg $NaNO_2$. The bright red reaction mixture was gently swirled for 2 hr at 0°C, the pH was adjusted to 7·0, and the conjugated protein was dialyzed against cold running tap water for 2 days. Lyophilization yielded a bright orange powder. Analysis of the conjugate by the anthrone method (Scott & Melvin, 1953) against a standard galactose curve indicated that 14 residues of sugar were combined per mole of BSA (assumed mol wt 70,000). The conjugated azo protein exhibited a visible absorption spectrum coincidental with that for an azobenzene-sulfonic acid conjugate of BSA reported by Higgins & Harrington (1959).

(c) *Immunochemical*

Rabbits were immunized with alum-precipitated preparations (Kabat & Mayer, 1948) of the antigen at a concentration of 1 mg/ml. Each rabbit received a total of 20 ml. in 16 intravenous injections; 4 injections per week for 4 weeks. These consisted of 4 injections each of 0·5 ml., 1·0 ml., 1·5 ml. and 2·0 ml.

The animals were bled by cardiac puncture 5 to 7 days following the last injection. Each animal was bled twice more by cardiac puncture, and then exsanguinated by severing the carotid artery approximately 14 days after the last injection. After separation of the sera the bleedings from a single rabbit were pooled. Merthiolate, final concentration 1 : 1000, was added as preservative.

All sera were absorbed with BSA before use. Addition of small amounts of BSA followed by centrifugation to remove precipitates was continued until a test on the supernatant with anti-BSA resulted in precipitation. At this point there was a slight excess of BSA, and all of the antibody capable of reacting with unconjugated BSA had been removed.

Quantitative precipitin curves were carried out as described by Kabat & Mayer (1948) using the Folin-Ciocalteu method (Heidelberger & MacPherson, 1943) for analysis of the specific precipitates. The ability of the various haptens to inhibit precipitation was determined by incubating the hapten with the antibody for 1 hr at 37°C prior to the addition of the antigen. The specific precipitate was analyzed colorimetrically and compared with a control, set up at the same time, containing only the equivalent concentrations of antigen and antibody.

The antigen solutions were standardized on the basis of their nitrogen contents as determined by a modification (Markham, 1942) of the micro-kjeldahl method.

Duplicate analyses were performed in all cases.

3. Results

Two of the sera obtained from individual rabbits immunized with galactoside-β-D-phenylazo-BSA were studied in detail. Quantitative precipitin curves obtained with these sera after absorption with BSA are depicted in Fig. 1. The amount of antigen required to give maximum precipitation, determined from these curves, was used for the hapten-inhibition studies. The results of these experiments with each serum are shown in Figs. 2 and 3.

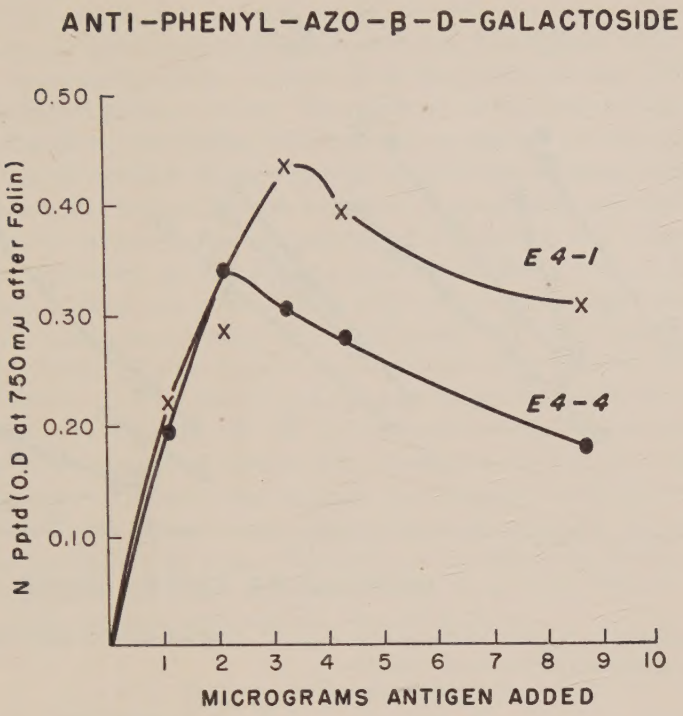

FIG. 1. Precipitin curves for the reaction between two absorbed anti-β-D-galactoside sera (E4–1 and E4–4) and phenylazo-β-D-galactosyl bovine serum albumin.

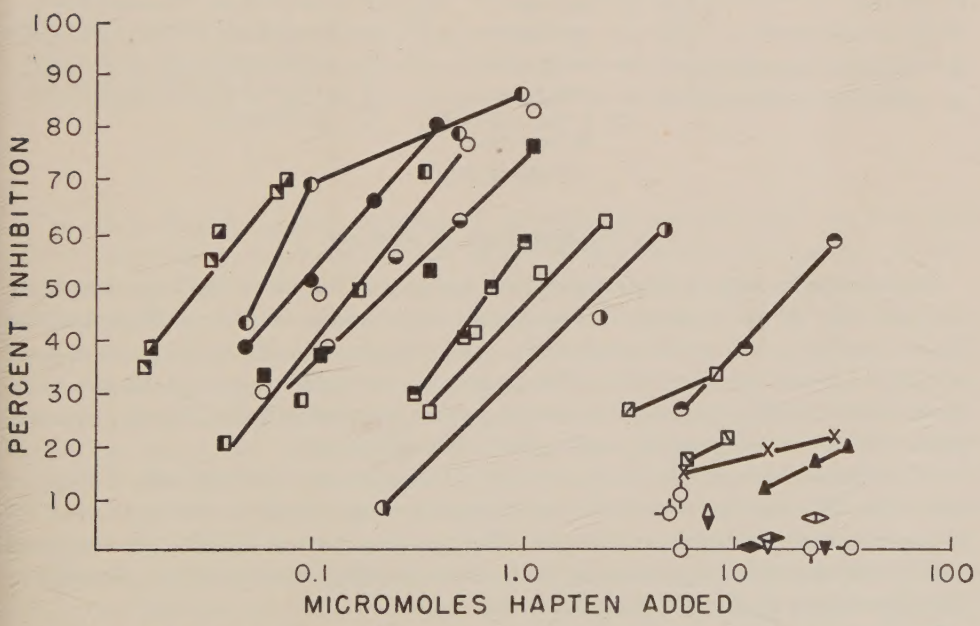

FIG. 2. Inhibition by haptens of the precipitation of absorbed anti-β-D-galactoside (E4–1) by phenylazo-β-D-galactosyl bovine serum albumin.

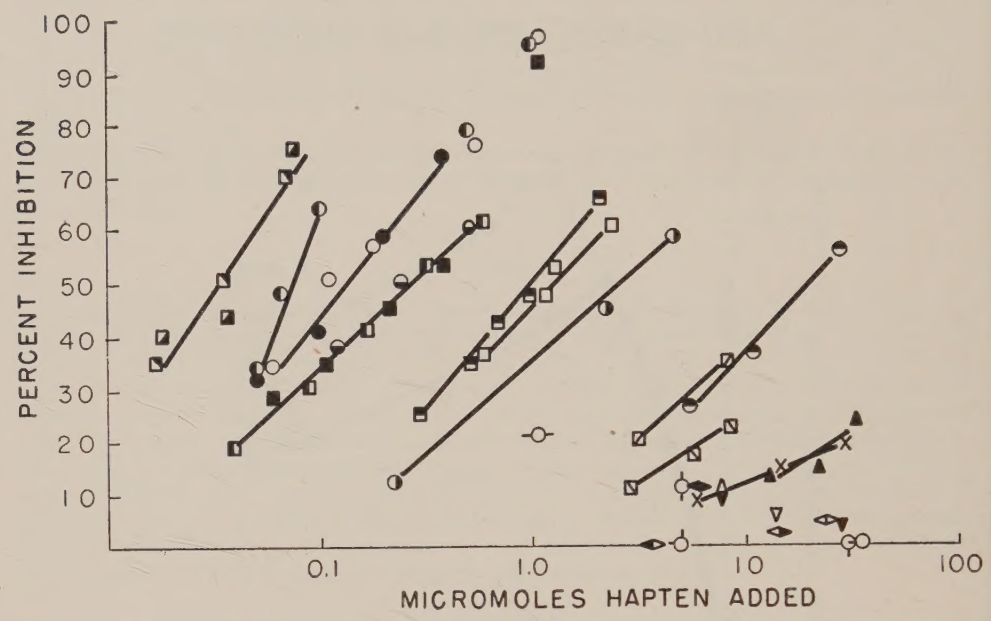

FIG. 3. Inhibition by haptens of the precipitation of absorbed anti-β-D-galactoside (E4–4) by phenylazo-β-D-galactosyl bovine serum albumin.

(a) *Specificity of antibody*

The specificity may be determined from the hapten-inhibition data by comparing the activities of the various monosaccharide and monosaccharide derivatives. The importance of L- and D-isomerism and α- and β-anomerism is shown by the lack of activity of L-galactose, and the ineffectiveness of *o*-nitrophenyl-α-D-galactoside (data not presented in Figs. 2 and 3; 0·7 μmole highest concentration tested) as a hapten compared with the corresponding *o*-nitrophenyl-β-D-galactoside.

Inversion of the hydroxyl group on carbon 4 in D-glucose results in lack of effective inhibition. The significance of the configuration around the 4th carbon is stressed by the finding that *p*-nitrophenyl-β-D-glucoside is a poor hapten inhibitor as compared with *p*-nitrophenyl-β-D-galactoside; and similar results were found for phenyl-β-D-thioglucoside *vs* phenyl-β-D-thiogalactoside.

The configuration of the hydroxyl group on carbon 2 is also important, since D-mannose, which differs from D-galactose at this position, is not an effective inhibitor.

Another relatively ineffective hapten is 2-deoxy-D-galactose, lacking a hydroxyl group at the 2nd carbon, and therefore unable to hydrogen-bond at this position.

The necessity for hydroxymethyl at carbon 6 was investigated using the pentose L-arabinose, whose configuration corresponds to that of D-galactose. This substance showed a moderate inhibitory activity. The specificity of this inhibition by L-arabinose is further indicated by the finding that D-arabinose did not exhibit any inhibitory activity. A limited number of experiments using 6-fluoro-6-deoxy-D-galactose, in which the hydroxyl group on the 6th carbon of D-galactose is replaced by fluorine, showed that this compound was comparable to D-galactose in inhibitory potency. The internal glycosides, β-1,6-anhydro-D-galactopyranoside and α-1,6-anhydro-D-galactofuranoside (data not presented in Figs. 2 and 3) were devoid of hapten-inhibitory activity at concentrations of 13 μmole and 20 μmole respectively. Failure of the former bicyclic anhydro sugar to combine with antibody must be due to conformational hindrance rather than to failure to hydrogen-bond with the group on the protein which bonds normally with the C-6 hydroxymethyl. This conclusion follows from the previously mentioned finding that L-arabinose can act, to a limited extent, as a hapten-inhibitor. It should also be noted that whereas all β-D-galactopyranosides studied combined with the antibody, ethyl-β-D-galactofuranoside did not.

Those disaccharides tested which contained a galactopyranosyl moiety, i.e. lactose, neolactose and melibiose, acted as haptens; while those which did not contain this grouping, namely cellobiose and maltose, were ineffective.

(b) *Size of the combining site*

The size of the combining site may be deduced from the data in Figs. 2 and 3 by noting the relative inhibitory activities of haptens which correspond to various portions of the introduced determinant group on the conjugated protein antigen. These substances fall into a graded series as follows: tyrosine-azophenyl-β-D-galactoside = histidine-azophenyl-β-D-galactoside > *p*-nitrophenyl-β-D-galactoside > phenyl-β-D-galactoside > *p*-aminophenyl-β-D-galactoside > methyl-β-D-galactoside > D-galactose. Essentially the same gradation is seen for the thio-analogues: *p*-aminophenyl-β-D-thiogalactoside > phenyl-β-D-thiogalactoside > methyl-β-D-thiogalactoside.

4. Discussion

The data from the quantitative hapten-inhibition experiments not only bear upon the specificity of the antibody, but may also be further interpreted to provide information on the size of the combining region, and on the heterogeneity of antibodies.

The specificity of the antibody for the β-D-galactopyranosyl structure is obvious, and these results confirm the observations originally made by Avery & Goebel (1929) using qualitative procedures. Since it was also found that stereochemical changes in the position of the hydroxyl group on carbons 2 or 4 destroyed hapten activity, one might speculate that D-gulose, which differs from D-galactose by an inversion at carbon 3, might be a poor hapten in this system. Of all the substituted monosaccharides tested, 6-fluoro-6-deoxy-D-galactose appeared to have activity as a hapten most nearly that of D-galactose. This is not surprising in light of the knowledge that in size, electronegativity, and ability to enter into hydrogen-bond formation, the fluorine atom approximates the hydroxyl group (Taylor & Kent, 1958; Pauling, 1945). It would appear that the specificity of the combining region in anti-β-D-galactosyl-antibody for D-galactopyranosyl has a complete requirement for

properly oriented groups capable of hydrogen bonding at carbons 2 and 4, and possibly at carbon 3; and a similar partial requirement at carbon 6.

The size of an antibody combining site has been studied most extensively by Kabat (1956, 1957, 1960) using the dextran-antidextran system. He found that this particular site, in most sera, corresponds to a structure complementary to a portion of the antigen the size of isomaltohexaose. The anti-β-D-galactoside system differs somewhat from Kabat's in that the antigen does not consist of a single repeating unit. Tyrosine-azophenyl-β-D-galactoside and histidine-azophenyl-β-D-galactoside were equally effective as inhibitors of anti-β-D-galactoside. It would seem, therefore, that this combining site corresponds to the galactosido-β-D-phenylazo fragment, but does not encompass the amino acid residue. It is furthermore known that the diazotization reaction with proteins occurs with both of these aromatic amino acids, and also with

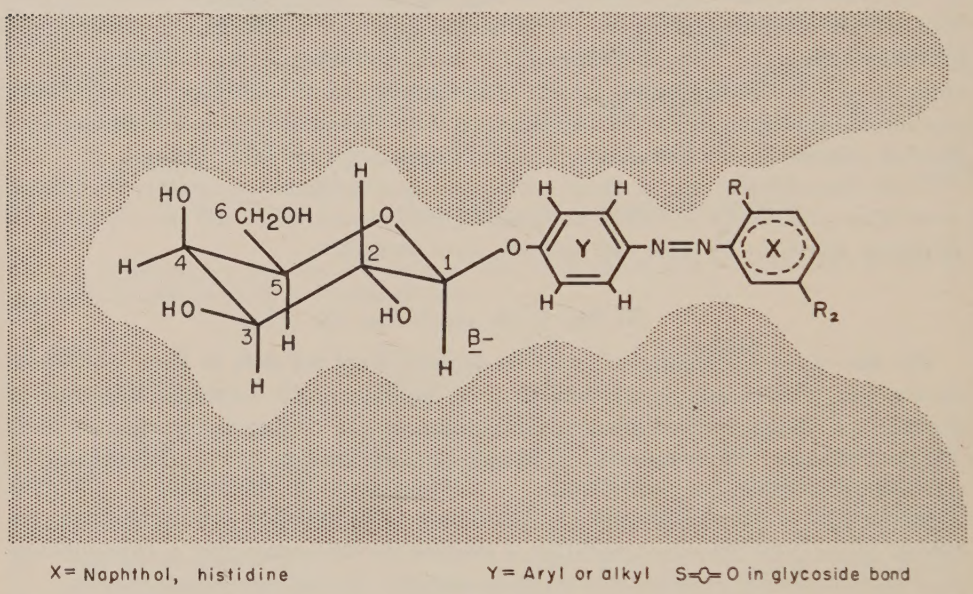

FIG. 4. Diagram of the combining site of anti-β-D-galactoside.

lysine, glycine, and perhaps with other residues (Gelewitz, Riedeman & Klotz, 1954; Howard & Wild, 1957; Higgins & Harrington, 1959). There is the possibility that these latter non-aromatic amino acids might also contribute to the determinant group. Additional experiments with α-naphthol-azophenyl-β-D-galactoside demonstrated that this hapten was as effective as the two aromatic amino acid azophenyl-galactosides. Since naphthol is not a constituent of proteins, and since its bulky structure is such that it might not be expected to fit with ease into a combining site specific either for tyrosine or histidine, it is probable that the combining site of the antibody is complementary to that portion of the determinant group which includes the galactopyranosyl, the *p*-orbital of the aromatic aglycone, and the azo moieties (see Fig. 4). Karush (1956) used a diazotized antigen for a physical-chemical study of the interaction between purified antibody and hapten. He also concluded that the antibody combining site probably corresponds to the entire introduced determinant group.

The proposed dimension for the site of anti-β-D-galactoside approximates the size calculated by Karush (1956), and encompasses roughly one-half the size reported by

Kabat (1960) for the human anti-dextran system. In all three systems the antibody site apparently corresponds to a branched chain in the antigen which occurs either as an artificially introduced determinant group, or may be naturally present as in dextran. (For discussion of dextran structure in relation to antibody *cf.* Kabat, 1960.) However it must be noted that the antibody studied here was carefully absorbed with bovine serum albumin before use, and perhaps this procedure left for study only antibodies with combining sites having an upper limit complementary to the determinant group.

A hypothetical representation of the combining site of the anti-β-D-galactosido-antibody is presented in Fig. 4. This schematic diagram shows the site as the conventional cavity in the surface profile of the protein. The finding that rabbit antibody has a single N-terminal amino acid (McFadden & Smith, 1955; Porter, 1950) suggests that such antibody consists of a single peptide chain, although this pattern may not be true for other species (Porter, 1948). If rabbit antibody is indeed a single peptide

TABLE 1

Relative molar concentrations of haptens required to inhibit precipitation with phenyl azo-β-D-galactosyl bovine serum albumin of two absorbed rabbit anti-β-D-galactoside sera by 50%.

Hapten	Relative concentration for 50% inhibition	
	Serum E4–1	Serum E4–4
Tyrosine-azophenyl-β-D-galactoside	1	1
Histidine-azophenyl-β-D-galactoside	1	1
p-Nitrophenyl-β-D-galactoside	2·0	2·1
Phenyl-β-D-galactoside	3·0	3·2
p-Aminophenyl-β-D-galactoside	4·0	3·8
p-Aminophenyl-β-D-thiogalactoside	6·0	7·9
Phenyl-β-D-thiogalactoside	8·0	7·9
o-Nitrophenyl-β-D-galactoside	8·0	7·9
*Iso*propyl-β-D-thiogalactoside	23	29
Methyl-β-D-thiogalactoside	37	38
Methyl-β-D-galactoside	90	84
D-Galactose	634	647

chain, the combining site could result from a juxtaposition of many folds of the chain, and a single three-dimensional site may contain contributions from amino acids that are not joined serially. This possibility is suggested by the recent X-ray data of Kendrew (1958) who has shown that the tertiary structure of myoglobin has a highly involved and convoluted topology around the prosthetic group. The proposal by Karush (1956) that the combining region consists of an interhelical cavity in the antibody globulin molecule might also involve amino acids that are not serially joined in the combining site.

The fine structure of the combining site in antibodies may also result from the 3-dimensional conformation and propinquity of two or more peptide chains held together by disulfide linkages. Nisonoff, Wissler & Woernley (1959) have suggested that two active fragments, each with an intact combining site, may be formed from antibody by breaking disulfide bonds. There may, however, be additional disulfide linkages required for maintaining the integrity of the combining site that are not

exposed and broken by the experimental procedure. Further experiments with highly purified antibody should help to resolve some of these problems of tertiary structure.

The concept of heterogeneity of antibodies which are formed in response to a single antigen is supported by several lines of evidence (Kabat, 1953). One experimental basis for this concept is that the molar ratios of haptens required for 50% inhibition of an antigen-antibody reaction vary from serum to serum. Kabat has used this type of argument to demonstrate heterogeneity of human antidextran (Kabat, 1957). If the hapten-inhibition data reported in Figs. 2 and 3 are treated in a similar manner and the haptens are all compared with tyrosine-phenylazo-β-D-galactoside, whose ratio is set at unity, the results in Table 1 are obtained. These ratios for each serum are remarkably similar despite their wide range. This close correlation indicates either that the two sera contain the same molecular species of antibody or the same spectrum of antibodies. This apparent micro-homogeneity may have resulted from the absorption procedure.

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Light Meromyosin Fraction I: A Helical Molecule from Myosin

Andrew G. Szent-Györgyi,† Carolyn Cohen‡ and Delbert E. Philpott

(*Received 11 April 1960*)

The fractionation of light meromyosin (LMM), obtained from myosin after short trypsin digestion, is described. About 50% of the total LMM is not denatured by ethanol. This portion, termed light meromyosin Fraction 1 (LMM Fr. 1), has a molecular weight of 120,000, behaves as a homogeneous material in hydrodynamic studies, and appears to be a single component on diethylaminoethyl-substituted cellulose columns. Optical rotation studies are compatible with the interpretation that this component of myosin is a fully coiled α-helix in solution. LMM Fr. 1 is further characterized by a 430 Å period in low-angle X-ray and electron microscope studies. The structure of the molecule and its relationship to myosin are discussed.

1. Introduction

During a study of the optical rotatory properties of fibrous α-proteins, we found that the light meromyosin (LMM) component of myosin, obtained after short trypsin digestion, could be further fractionated into a component which is not denatured by ethanol and which has different optical activity from the starting material (Cohen & Szent-Györgyi, 1957).

This material, called light meromyosin Fraction 1 (LMM Fr. 1) behaved as a fully coiled α-helix, and could be obtained in yields of about 50% of LMM. In this paper, the preparation and some of the properties of LMM Fr. 1 will be described. New observations on LMM will also be reported.

(a) *Isolation of LMM Fr.* 1

LMM Fr. 1 has a solubility very similar to LMM and to myosin in neutral salts of alkali metals or ammonium sulfate. Selective denaturation by the use of non-polar solvents is necessary for the removal of these other proteins.

Procedure A. Starting material is a three times reprecipitated (or washed) LMM obtained after 10 to 12 min digestion of myosin with trypsin at a weight ratio of 1 : 200 (Szent-Györgyi, 1953). To the 0·6 M-KCl solution, 3 vol ethanol (95% v/v) are added at room temperature and the mixture is allowed to stand for 2 to 3 hr. The protein is quantitatively precipitated and collected by centrifuging at 2000 rev/min. The precipitate is resuspended

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‡ The Children's Cancer Research Foundation, and The Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. This investigation was supported in part by N. I. H. Grant No. A-2633.

in the initial volume of 0·6 M-KCl, containing 0·01 M-phosphate buffer, pH 7·0, and dialyzed against 10 vol of the same solution to reduce the ethanol concentration. LMM Fr. 1 redissolves in 0·6 M-KCl; the rest of the protein remains insoluble. The supernate is precipitated by dialyzing against 12 vol 0·01 M-phosphate buffer, pH 6·5. The protein precipitates invariably in the form of needle-shaped paracrystals (Plate I). "Recrystallization" is repeated 3 times by dissolving in 0·6 M-KCl at neutral pH and reprecipitating at low ionic strength. Acetone can replace ethanol in the procedure since LMM Fr. 1 is not denatured by this solvent. The yield is about 50 to 60% of the initial LMM. This yield was unaffected by ethanol treatment for 24 hr.

Procedure B. LMM Fr. 1 may be prepared directly by treating digested myosin with ethanol. The following steps are similar to those in *Procedure A*, with the addition that centrifugation is required at 15,000 rev/min when LMM Fr. 1 is first redissolved in 0·6 M-KCl. (This step removes aggregated material.) From this procedure, the yield is 15 to 17% of myosin.

A similar fraction was recently obtained by means of a procedure where ethanol treatment preceded trypsin digestion (Laki, 1957).

Preliminary experiments indicate that LMM obtained by digestion of myosin with chymotrypsin or subtilisin also contains an ethanol-resistant fraction, with properties apparently similar to the fraction obtained after trypsin digestion.

(b) *Production of LMM Fr. 1 during digestion*

The ease of separation of LMM Fr. 1 from myosin enables one to measure production of LMM Fr. 1 during the course of tryptic action. From the change of viscosity, one

TABLE 1

Production of LMM Fr. 1 during digestion

Time of digestion min†	η_{rel} of 2 × diluted solution	Myosin digested mg/ml.¶	LMM Fr. 1‡ formed mg/ml.	LMM Fr. 1§ formed mg/ml.	Myosin digested %	LMM Fr. 1‡ formed % of max ‖	LMM Fr. 1§ formed % of max ‖
Blank	2·025	0	0·04	0·06	0	0	0
0	2·05	0	0·15	0·14	0	0	0
0·5	1·935	0·83	0·35	0·26	13	19	11
1·0	1·816	1·75	0·42	0·40	27	26	24
2	1·667	2·97	0·64	0·61	45	47	44
4	1·515	4·33	0·82	0·87	66	64	68
8	1·355	5·95	1·09	1·09	91	90	89
12	1·307	6·48	1·18	1·21	99	99	100
20	1·243	(7·18)	1·19	1·16	(109)	(100)	95

† To 20 ml. 0·787% myosin, dissolved in 0·6 M-KCl and 0·067 M-phosphate buffer, pH 7·0, 2 ml. 0·05% trypsin was added and the reaction was stopped at various times by addition of 2 ml. 0·1% soybean trypsin inhibitor. ‡ Procedure A (Kjeldahl). § Procedure B (Kjeldahl).

‖ Expressed as percentage of maximum difference between the values at complete digestion and zero time. ¶ Calculated from viscosity.

can estimate the amount of myosin which was fragmented during trypsin treatment. This is shown in Table 1. After digestion was stopped at various times, a portion was dialyzed against low salt to precipitate LMM and myosin. The precipitate was redissolved, treated with ethanol and LMM Fr. 1 was prepared (*Procedure A*). Table 1 shows the protein recovered after the first crystallization. Another portion of the digest was treated directly with ethanol and LMM Fr. 1 was prepared (*Procedure B*). Again, the protein yield from the first crystallization is shown in Table 1. If the

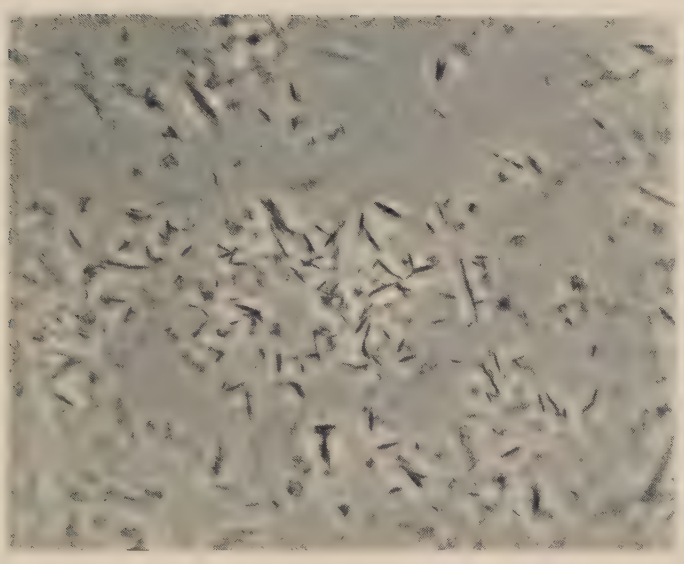
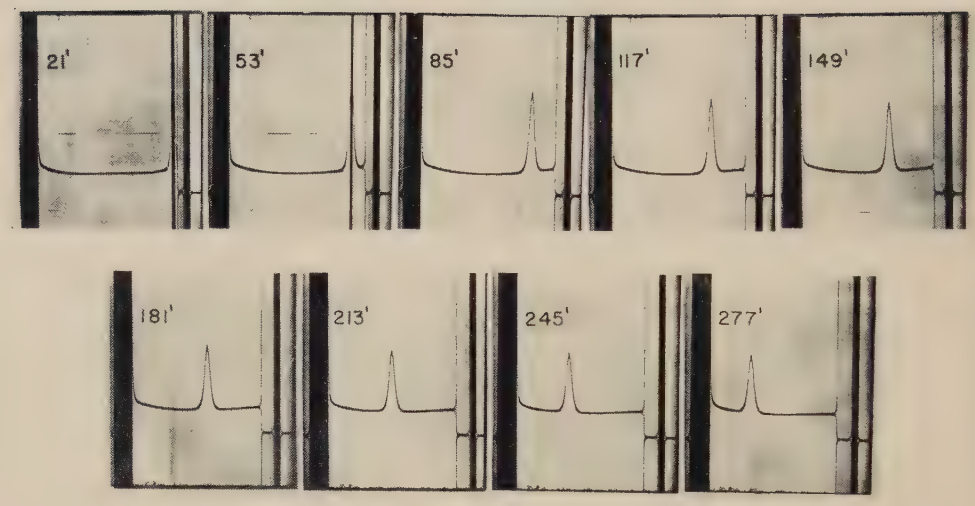
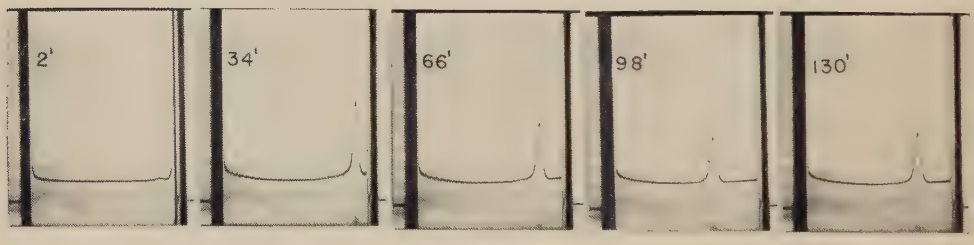


PLATE I. Paracrystals of LMM Fr. 1 in phase microscope. (Magnification × 800.)

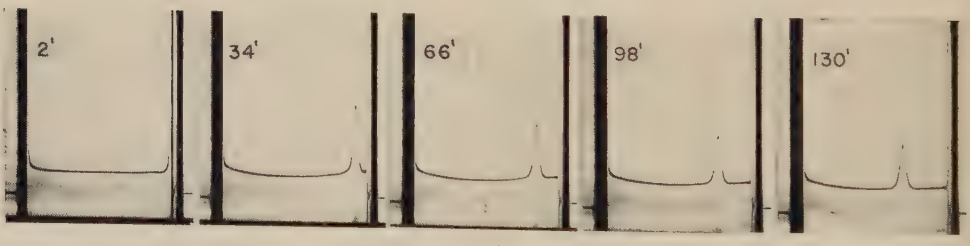
[*To face page* 134



(a)



(b)



(c)

PLATE II. Sedimentation patterns of LMM Fr. 1, LMM and their mixture.

(a) 0·8% LMM Fr. 1.

(b) 0·54% LMM.

(c) a mixture of equal weights of LMM Fr. 1 and LMM.

Bar angle 70°, 59,790 rev/min, pictures taken at 32 min intervals. Sedimentation proceeds to the left. Solvent 0·6 M-KCl and 0·01 M-phosphate buffer, pH 7·0.

amount of LMM Fr. 1 obtained is expressed in percent of the maximum of the LMM Fr 1, then the yields at different stages of digestion are closely proportional to the amount of myosin broken down. This experiment indicates that there is no obvious delay in the formation of LMM Fr. 1 and its quantity, to a first approximation, depends directly on the amount of myosin digested.

2. Physico-Chemical Characterization

(a) *Sedimentation, viscosity and molecular weight of LMM Fr.* 1

Both LMM, LMM Fr. 1, and their mixture, sediment as an apparently homogeneous material with a single symmetrical peak (Plate II). Thus, any molecular weight difference between LMM and LMM Fr. 1 is not great enough to be resolved in this type of sedimentation pattern. The sedimentation of LMM Fr. 1 extrapolates to about 3·0 and has the same slope as that of LMM.

The intrinsic viscosity of LMM Fr. 1 is 0·9 in 0·6 M-KCl at neutral pH. This value may be compared with the intrinsic viscosity of 1·0 obtained for LMM.

The molecular weights of three different preparations of LMM Fr. 1 were measured, using the Archibald method (Plate III). Table 2 shows the values obtained after different times of centrifugation. A partial specific volume of 0·728 was calculated from amino acid content. The molecular weight values remained constant during the run, indicating the homogeneity of the preparation. The molecular weight averaged about 120,000, which is higher than that reported previously for LMM based on sedimentation-diffusion data, but agrees well with the more recent determinations (Lowey & Holtzer, 1959).

TABLE 2

Molecular weight of LMM Fr. 1 *and of protomyosins obtained from the approach to sedimentation equilibrium*

Preparation	Conc. mg/ml.	Area	Rev/min	Time in min	Mol wt†	Average
LMM Fr. 1						
1	6·1	0·04894	8,660	25	122,400	
				96	124,500	
				237	120,200	
				1256	112,500	
				1526	115,700	
				1936	122,500	119,600
2	7·0	0·05621	6,353	17	128,400	
				33	131,200	
				61	118,900	
				130	121,600	
				190	110,400	
				250	116,400	
				595	117,300	120,600
3	6·3	0·05015	6,353	16	132,800	
				49	113,500	
				141	120,000	
				222	124,000	
				252	125,600	123,200

TABLE 2 (*contd.*)

Preparation	Conc. mg/ml.	Area	Rev/min	Time in min	Mol wt†	Average
Protomyosin (LMM Fr. 1 16 hr in 5 M-urea, then dialyzed against 0·1 M-KCl and 0·067 M-phosphate buffer, pH 7·0)						
1	7·8	0·06282	20,410	17	7,261	
				33	6,726	
				49	6,646	
				107	6,558	
				139	5,724	
				171	5,422	
				203	5,032	
				235	5,027	
2	4·9	0·03931	24,630	16	10,900	
				32	11,300	
				48	11,470	
				64	11,250	
				80	12,010	
				96	11,500	
				112	10,750	
				128	11,131	
				144	10,455	

† Calculated from the meniscus.

In urea LMM Fr. 1 depolymerizes into small components, protomyosins, similarly to LMM (Szent-Györgyi & Borbiro, 1956). The protomyosins have a low molecular weight of about 5,000 to 10,000 (Table 2), even after removal of urea, and are soluble at low ionic strengths where LMM Fr. 1 precipitates.

These physico-chemical measurements indicate that LMM and LMM Fr. 1 are very similar in shape and molecular weight.

(b) *Cellulose column experiments*

In the previous section, hydrodynamic studies were described which gave values for molecular size and shape of LMM, without yielding definitive information about homogeneity. In fact, LMM from which LMM Fr. 1 is isolated gave no evidence of heterogeneity by the above techniques. Therefore, homogeneity was further tested with the aid of diethylaminoethyl (DEAE) substituted cellulose columns.

DEAE cellulose was obtained from Eastman Kodak Company, cleaned according to Peterson & Sober (1956) and washed with 1 M-NaCl. $1{\cdot}7 \times 20$ cm columns were filled under 5 to 7 lb/sq in pressure and eluted at the rate of 1 drop in 10 to 15 sec in the cold (1 to 4°C). Runs were made at pH ranges of 6·8 to 9·0; 0·02 M-tris (2-amino-2-hydroxymethylpropane-1 : 3-diol) buffer was used for pH 8, 8·5 and 9·0, and 0·02 M-phosphate for the more acid pH's. The gradient was applied by the dropwise addition of 0·5 or 1·0 M-NaCl solution containing the appropriate buffers of the same pH and concentration. At low ionic strength, LMM Fr. 1 dissolved only at pH values of 8·0 to 9·0. Between pH 7 and 8 the protein is insoluble at low ionic

strength. To insure uniform solubilization the precipitate was formed by dialysis, applied directly without centrifugation, and stirred with the upper 2 cm layer of the cellulose.

At all pH values, LMM Fr. 1 came off as a single peak with no apparent shoulder on either side (Fig. 1(a), (b), (c)). As alkalinity increased, the ionic strength at which the protein appeared was shifted slightly to lower values. In comparable runs, LMM gave indications of at least three components: a main peak in position corresponding to the LMM Fr. 1 and amounting to about 50 to 60% of the protein, a faster component of 7 to 8% (which may be heavy meromyosin (HMM) contamination since it did not precipitate when the ionic strength was lowered), and finally, a broad shoulder which amounted to 35 to 45% of the total protein (Fig. 1(d), (e)). The main peak and the shoulder precipitated at low ionic strength at neutral pH, and formed needle-shaped paracrystals having a characteristic periodicity in the electron microscope as described in the next section.

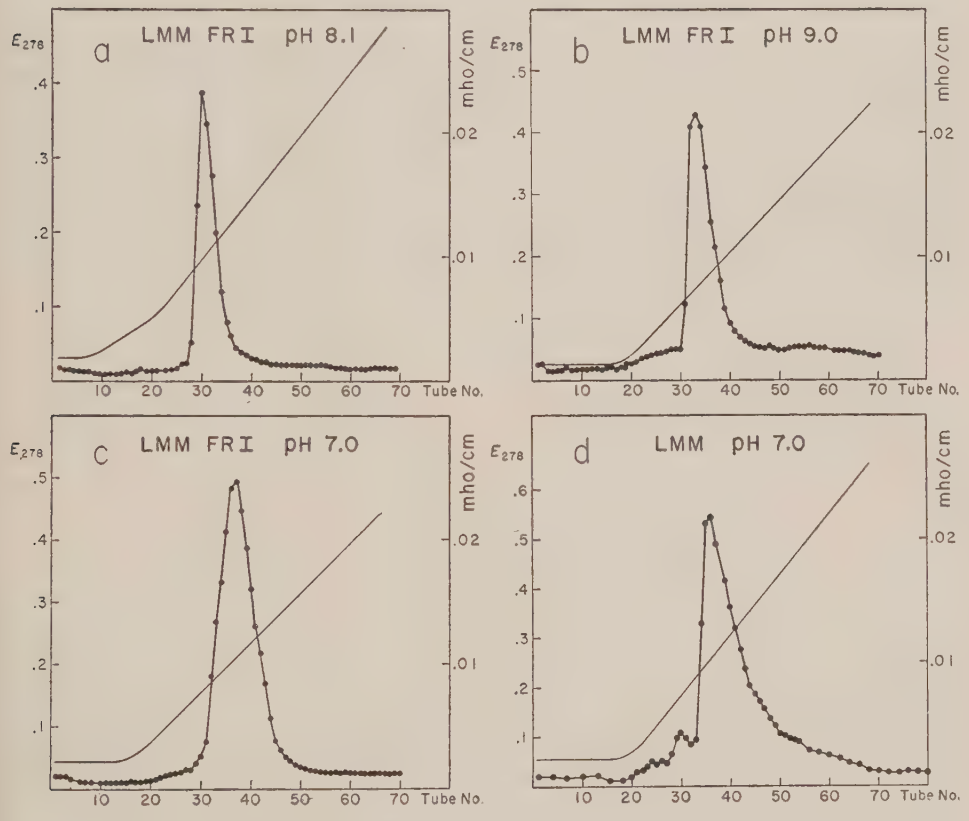


Fig. 1. DEAE chromatography of LMM Fr. 1 and LMM. Protein put on in 0·02 M-phosphate or tris buffer of appropriate pH and gradient elution applied by the dropwise addition of a 1 M-NaCl solution of the pH to the mixing chamber. (Column size, 1·7 × 20 cm; drop rate, 10 to 15 sec/ drop; volume, 4 ml. per tube.)

Left hand ordinate, extinction; right hand ordinate, specific conductance.

With LMM Fr. 1 the recovery was 90 to 95% of the applied protein. The impurity which is retained by the column, at the ionic strength used, appears to be nucleic acids which are present in LMM preparations and co-precipitate with LMM Fr. 1. Thus, LMM Fr. 1 prepared by repeated re-precipitations shows an extinction of 0·380

at 278 mμ in 0·1% concentration and 1 cm path-length. The protein concentrations were based on Kjeldahl nitrogen determinations using a factor of 6·42 calculated from amino acid analysis. The ratio of extinctions at 278 and 260 mμ is 1·44. After fractionation on the column, the extinction changes to 0·240, and the ratio to 1·80. At more acid pH's the precipitation zone extends to higher ionic strengths preventing ion exchange chromatography.

(c) *Large periodicity*

LMM paracrystals are characterized electron optically by a 420 Å repeat period, visible without staining or shadow-casting (Philpott & Szent-Györgyi, 1954). The electron microscope picture of LMM Fr. 1 is very similar. Intense lines are separated by 420 ± 25 Å and between the main periods five much fainter lines can be seen (Plate IV(a)). The subperiods are best observed when paracrystals are allowed to dry on the grid and are stained for a few minutes with 0·1% PTA (phosphotungstic acid) at pH 5·4. If KCl is omitted from the PTA solution, or the paracrystals are washed with water after staining, the main periodicity is missing, and only subperiods can be seen (Plate IV(c)). Accumulation of monovalent cations at specific regions is very likely the cause of the enhancement of the 420 Å electron microscopic repeat.

TABLE 3

Small-angle meridional and near-meridional reflections of light meromyosin

d (Å)	I	Layer line index k	period (Å) $k \times d$
143	s.	3	429
70·8	v.s.	6	425
47·6	w.	9	430
43·0	w.	10	429
39·0	w.	11	429
35·9	w.	12	430
26·8	m.	16	429
23·8	w.	18	428
21·3	w.	20	426
15·9	m.	27	429

Oriented fibers of LMM and LMM Fr. 1 were prepared for X-ray diffraction by the following procedure. The protein paracrystals were precipitated by dialysis against water, whose pH was maintained between 7 and 8 by the addition of 0·01% $NaHCO_3$. At ionic strengths less than about 0·01, the paracrystals swelled and formed a transparent gel. (In the electron microscope, the dried gel appears as a network of long filaments, about 100 to 200 Å in width.) The gel was oriented between forceps and allowed to air-dry. The resulting transparent fiber had good orientation. Fibers prepared from both LMM and LMM Fr. 1 yield a characteristic small-angle pattern, but the degree of crystallinity varied widely in different preparations. The best pattern was obtained from an LMM sample and showed about 10 orders of a 428 Å repeat (Plate V, Table 3). A pattern obtained from LMM Fr. 1, while having poorer orientation, seemed identical with that of LMM with regard to the strong orders. We note that in both the electron micrographs and the X-ray patterns a strong 70 Å spacing is present.

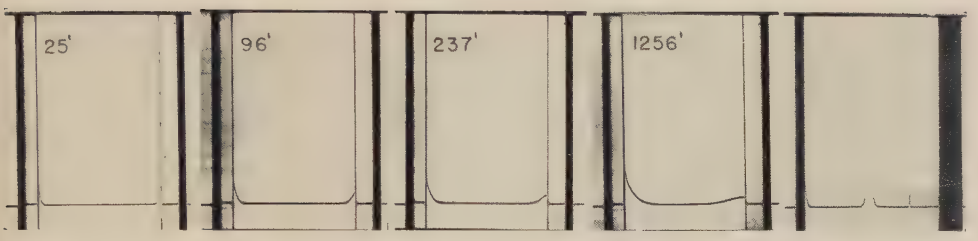
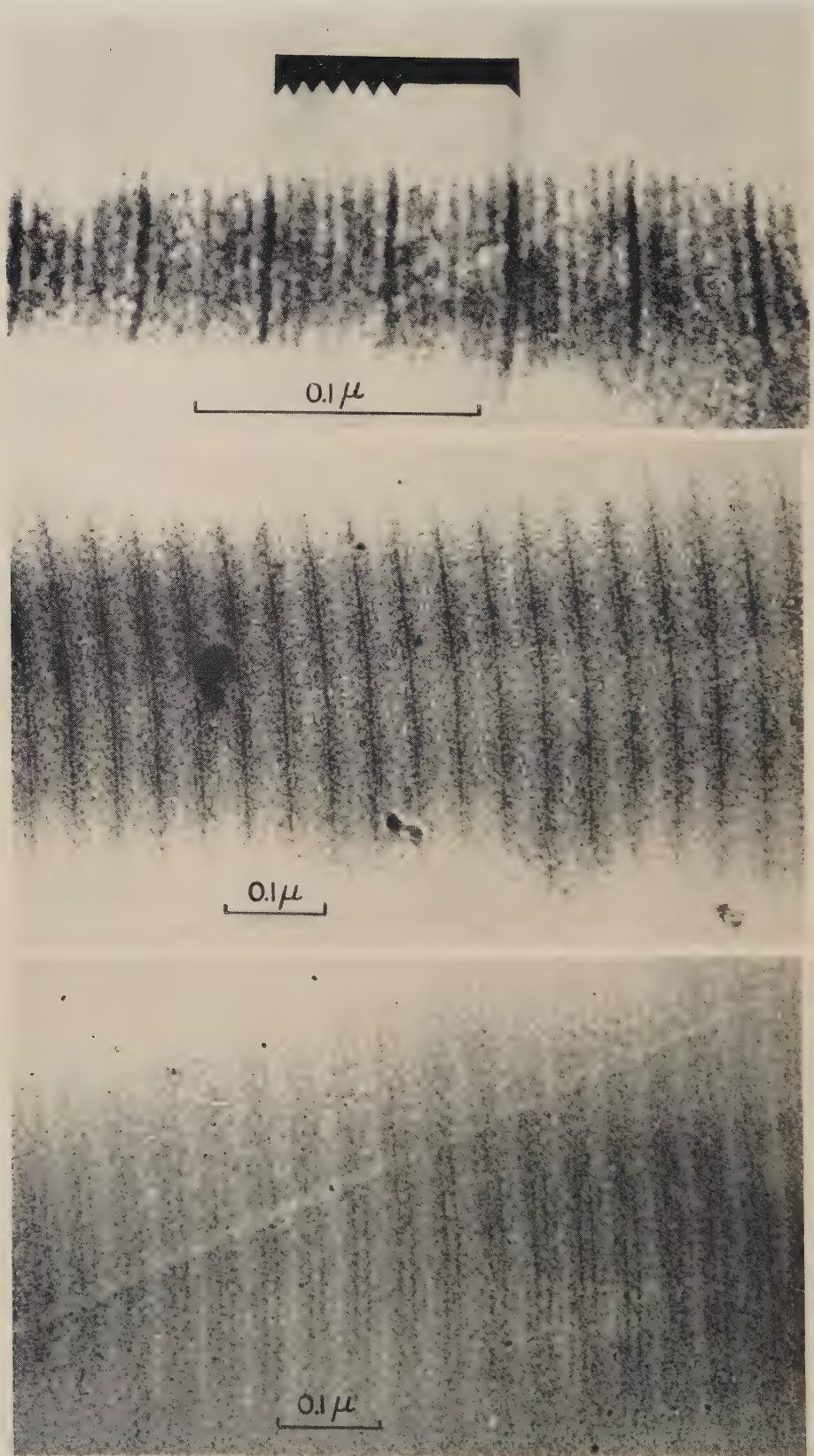


PLATE III. Patterns of Archibald runs of LMM Fr. 1, 8,600 rev/min. Meniscus at the right-hand side of the patterns. Bar angle 80°.



(a)

(b)

(c)

PLATE IV. Electron microscope pictures of LMM Fr. 1, stained with PTA at pH 5·4.
(a) and (b) in presence of 0·04 M-KCl, (c) in absence of salt (see text). Main periodicity 420 to 430 Å, fine periodicity 70 Å.

PLATE V. X-ray diagrams of LMM fibers. Insert: low angle photograph showing 143 Å and 70·8 Å spacings.

It is important to emphasize that the similarities between LMM and LMM Fr. 1 may not necessarily mean that these molecules have, in fact, identical properties. Thus both the electron microscope and X-ray periodicities of LMM may arise from its LMM Fr. 1 content.

(d) *Optical rotation and polypeptide chain configuration of LMM Fr. 1*

LMM and LMM Fr. 1 gave an α-pattern by wide-angle X-ray diffraction, and the diagrams indicate that these molecules contain large regions of α-helices presumably in some coiled-coil arrangement.

One can obtain estimates for the α-helix content of these proteins in solution by application of the Moffitt treatment for rotatory dispersion (Moffitt, 1957; Moffitt & Yang, 1956). Moffitt plots

$$(m)\times(\lambda^2-\lambda_0{}^2) \textit{ vs } 1/(\lambda^2-\lambda_0{}^2)$$

of the dispersion of these proteins are shown in Fig. 2. m is the average residue rotation, λ_0 is the dispersion constant and was taken as 2100 Å. (For reference, the rotatory dispersion of myosin and heavy meromyosin are included on the figure.) From the

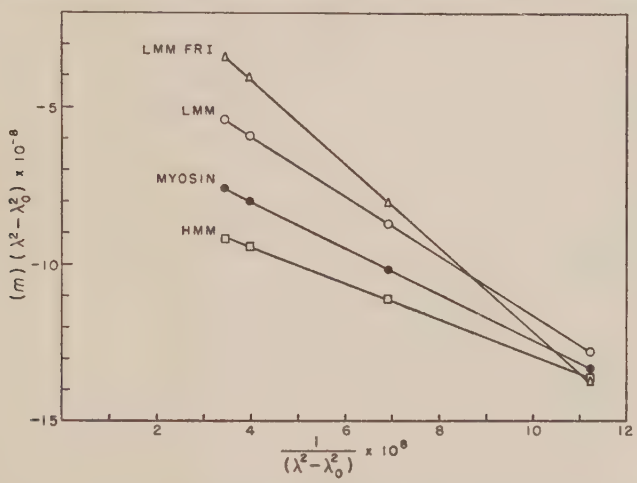


Fig. 2. Moffitt plots of LMM Fr. 1, LMM, HMM and myosin.
Measurements in 0·6 M-KCl and 0·01 M-phosphate buffer, pH 7·0 at room temperature.

slopes of these lines a constant, b_0, is obtained. b_0 is assumed to be proportional to α-helix content and is −660° for LMM Fr. 1 and −490° for LMM. LMM Fr. 1 has, in fact, one of the highest negative b_0 values of a number of α-proteins we have studied, a feature which has been correlated with its low proline content (Szent-Györgyi & Cohen, 1957). The rotatory dispersion data are consistent with the interpretation that this protein behaves as a fully coiled α-helix in aqueous solution. In contrast, the helix content of LMM was calculated to be about 74%, based on the relative ratio of its b_0 value to that of LMM Fr. 1 (Szent-Györgyi & Cohen, 1957). The stability of the LMM Fr. 1 helical configuration is great; for example, acidification to pH 2 resulted in an increase of less than 10% in the specific rotation.

3. Discussion

(a) *Biological aspects*

The properties of myosin are strikingly divided between the meromyosins, HMM having all the ATPase activity and actin-binding capacity of myosin, and LMM

having the same solubility as myosin. The meromyosins have been used to clarify problems such as the topography and synthesis of myosin. Thus the exchange studies of Velick showed that myosin had unique features consistent with an independent turnover rate for LMM and HMM (Velick, 1956). Immunological analysis of myosin also showed unusual features in that 4 distinct types of antigenic sites were observed (Marshall, Holtzer, Finck & Pepe, 1959). Of these, one was uniquely present in LMM and another in HMM. LMM Fr. 1 is the only immunologically homogeneous subunit containing the site characteristic for LMM. This property of LMM Fr. 1 permits its use as a relatively reliable marker in the myosin subunit localization in the myofibril (Marshall *et al.*, 1959).

(b) *Interpretation of enzymatic action*

In order to obtain meromyosins, digestion by a proteolytic enzyme (e.g. trypsin) is required. It is important to determine the degree to which enzymatic attack is reflected in the structure of the molecules. That LMM Fr. 1 is a single molecular species is shown by the immunological homogeneity, the sedimentation pattern and, most strikingly, by the DEAE column studies. Upon urea treatment LMM Fr. 1, similarly to LMM, breaks down into units of molecular weight approximately 5,000. One might expect that these "protomyosins" are a direct result of the trypsin treatment since myosin before proteolytic attack does not depolymerize into small units in a single step. However, we note that the total number of end groups present in a digest, as indicated from proton liberation studies (Mihalyi & Harrington, 1959), is far more than those present in the HMM and LMM isolated from such a digest (Middlebrook, 1959) (measured with fluorodinitrobenzene); and that the C-terminal end groups obtained from the meromyosins do not correspond to trypsin specificity (Middlebrook, 1959). Hence in the isolation of the meromyosins there must be a fractionation which removes material containing end groups and favors "intact" components.† In further confirmation of the concept that meromyosins represent relatively intact portions (hence "subunits" of myosin) the optical rotation shows very little change during the initial production of these molecules (Cohen & Szent-Györgyi, 1957). Thus in considering the protomyosins one concludes either that they are not present in native myosin so that the specificity of trypsin is unusual in the case of this molecule, or that the protomyosins are present in myosin, and trypsin increases their availability to reagents such as urea.

(c) *Origin of LMM Fr.* 1 *and its relation to myosin*

Although the procedure for obtaining LMM Fr. 1 is quite simple, its relation to the original myosin molecule is not yet clear. Two possibilities may be considered. (1) Both LMM Fr. 1 and another molecule of similar size are present in a roughly mole to mole ratio in one myosin molecule. Considering the sedimentation area determinations (giving the weight ratio of HMM to LMM as about 70 to 30) (Holtzer & Lowey, 1956) this picture is possible only if the molecular weight of myosin is taken as 620,000 (Kielley & Harrington, 1960) rather than the previously reported 400,000 to 500,000 (Holtzer & Lowey, 1956; Gergely, Kohler, Ritschard & Varga, 1958; von Hippel, Schachman, Appel & Morales, 1958; Mommaerts & Aldrich, 1958). The latter

† We do not know whether the material containing large numbers of end groups is present in some restricted areas (amorphous region) or represents some highly digested meromyosins. The total amino acid composition of the meromyosins adds up quite well to that of myosin (Kominz, Hough, Symonds & Laki, 1954); thus, at present, we favor the latter picture.

molecular weight contradicts the concept that myosin contains two species of LMM molecules. (2) The differences between LMM and LMM Fr. 1 are due to some "peptide" material present, either originally in the myosin molecule, or added subsequent to the trypsin attack. A comparison of the amino acid compositions of LMM (Kominz *et al.*, 1954) and LMM Fr. 1 (Laki, 1957) is consistent with the latter concept. We note, for example, that all the amino acids found in LMM Fr. 1 are also present in LMM. Some of the amino acids of LMM are missing, and are greatly decreased in LMM Fr. 1. The major differences are the great decrease in cysteine, proline and phenylalanine in LMM Fr. 1. The size of the extra peptide is limited by the accuracy of the molecular weight determinations and one would not expect it to be larger than about 10,000 to 20,000.

(d) *A model for LMM Fr.* 1

From the preceding experimental data and discussion we may derive a schematic model for LMM Fr. 1. From the amino acid content and molecular weight one calculates the length of a two- or three-chain α-helical model as 810 Å and 540 Å, respectively. Assuming 25% hydration, an intrinsic viscosity of 1·0 corresponds to an asymmetry of 1 : 37 and a prolate ellipsoid with length about 750 Å. Thus, from present experimental data a two-chain model seems most probable. It is clear, however, that viscosity data yield only a very approximate molecular length, and light-scattering studies on this molecule would be definitive in the choice between the two- or three-chain unit.

The "subunits" of LMM Fr. 1 are the population of protomyosins consisting of roughly 50 amino acid residues each. On the two, or three, helically wound chains the ends of these subunits occur in some staggered fashion, and are not available to reagents before urea treatment. The amino acids on the chains must bear a special relation one to the other, which insures stability of the α-helical configuration in aqueous solutions over a wide pH range. Thus, the various protomyosins would be expected to have sequences favoring "knobs into holes" packing of side chains on adjacent helices (Crick, 1953). The precise nature of α-helical stabilization awaits further sequence analysis but it is likely that hydrophobic bonds between side chains are of major significance and that polar groups are exposed to the environment (Crick, 1953; Waugh, 1954; Kauzman, 1959).

There are a great many problems which remain to be answered at this time: the exact length of LMM Fr. 1 (hence number of chains); the exact molecular weight of myosin (hence number of LMMs present); why the protomyosins can be liberated by urea only after tryptic treatment; and finally, the relationship of LMM Fr. 1 to LMM. Nevertheless, the isolation of LMM Fr. 1 demonstrates that a homogeneous, highly helical, stable molecule can be readily produced from myosin.

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Molecular Homogeneity of the Deoxyribonucleic Acid of Phage T2 †

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Chromatographic analysis by means of a column of basic protein is used to measure the breakage of T2 DNA by stirring. Broken molecules elute from the column at lower salt concentrations than do the original molecules. At a critical low speed of stirring, one can produce single breaks near the centers of the molecules, as shown by the all-or-none character of the initial change in chromatographic behavior, and by the survival of unbroken molecules according to an exponential function of time of stirring. In an analogous way, one can produce fragments resulting from three breaks per molecule, and in general stirring for a long time at a given speed produces a moderately homogeneous collection of fragments whose mean size is smaller, the higher the speed of stirring.

The sensitivity of the DNA to breakage by stirring is strongly dependent on the concentration of DNA, being greater the lower the concentration. This self-protective action is greatly reduced when the DNA is broken by stirring.

By chromatographic refractionation of the fragments produced by initial breaks, it can be shown that the fragments are not identical in chromatographic properties, presumably because of a continuous distribution of lengths centered about the mean half-length. Unbroken DNA, on the contrary, is homogeneous in chromatographic properties, therefore presumably homogeneous in molecular length. We conclude that DNA can be extracted from T2 by phenol without manipulative breakage, and that it exists in the phage particle in the form of one or more molecules of identical length.

1. Introduction

Existing methods for the measurement of molecular weights and molecular weight distributions of deoxyribonucleic acids are of doubtful significance at best and may be inapplicable when the molecular weight becomes very high. This is so both because the methods are theoretically ambiguous or technically complicated or both, and because the nucleic acids are prone to preparative degradation. The resulting confusion is well illustrated by the status of the DNA of T2, which is variously reported to consist of a mixture of larger (molecular weight 40 million) and smaller pieces (Levinthal & Thomas, 1957); to be homogeneous at a molecular weight about 14 million (Meselson, Stahl & Vinograd, 1957); and to be composed of two fractions differing both in composition (Brown & Martin, 1955) and in molecular size (Brown & Brown, 1958).

We have re-examined the DNA of T2 at some length by chromatographic and ultracentrifugal methods that did not, at the outset, seem particularly auspicious. We now conclude, however, that both methods are exceedingly useful for the analysis

†Aided by grant C2158 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

of nucleic acids of high molecular weight; that the molecular weight of T2 DNA is indeed high; and that undegraded preparations homogeneous in molecular weight can be made.

In this paper we present evidence concerning the homogeneity of our preparations, based on the following argument. Samples of DNA that have been stirred for a long time at a given speed may be expected to consist of fairly uniform fragments whose average molecular length will be shorter the higher the speed of stirring. This expectation is borne out by the fact that such samples elute in a single band from a column of basic protein at a salt concentration that is lower the higher the speed of stirring. Nevertheless, DNA broken by stirring can easily be resolved into chromatographically distinguishable components by chromatographic separation. Unstirred DNA, on the contrary, is chromatographically homogeneous, except that the samples usually contain a small and variable fraction of broken molecules.

It will be noticed that our present argument rests solely on the application of chromatographic methods, which perhaps leave something to be desired. In a paper to follow (Burgi & Hershey, in preparation) we shall extend the analysis in other ways.

2. Methods

DNA is prepared from phage T2 by shaking the purified phage suspensions with water-saturated phenol (Mandell & Hershey, 1960). The resulting preparations contain 0·4 mg of DNA per ml. in a solution 0·1 M in NaCl and 0·1 M in sodium and potassium phosphate of pH 7·1.

Chromatographic analysis is made by means of a column of methyl-esterified serum albumin adsorbed to diatomaceous earth (Mandell & Hershey, 1960). About 0·4 mg of DNA in 50 ml. of 0·66 M-NaCl is applied to the column, from which the DNA is eluted again (at about 0·72 M) under a gradient of rising salt concentration. The salt solutions are buffered at pH 6·7 with 0·05 M-phosphate. In the illustrations shown in this paper, "tube number" means the number of 3·6 ml. samples that have passed through the column behind the gradient front under an approximately constant gradient of, usually, $3{\cdot}3 \times 10^{-4}$ M/ml.

The chromatographic analyses are of two kinds. "Chromatographic separations" are made by passing material through a column and collecting fractions in the usual way. For "chromatographic comparison", two samples of DNA, one labeled with radiophosphorus and one not, are mixed in such proportions that the labeled sample does not contribute appreciably to the optical density of the mixture. The mixture is then passed through a column equipped for continuous recording of the ultraviolet absorbency and radioactivity of the effluent, which permits the labeled and unlabeled samples of DNA to be compared with great precision. The results obtained in this way are then converted into percentage distributions of DNA among the hypothetical 3·6 ml. tubes. This method has about the same accuracy as actual collection and assay of fractions.

Intentional breakage of DNA molecules is achieved by stirring for specified times at specified speeds and concentrations as follows. For high speeds (>600 rev/min) we use a Virtis Co. (Yonkers, New York) stirring blade and 5 ml. flask attached to a powerful series-wound motor. Lower speeds are obtained with a larger Virtis stirrer and 50 ml. flask attached to a gear-reduction motor capable of speeds between 100 and 600 rev/min. The speed of both motors is controlled by a variable transformer and voltmeter.

3. Results

(a) *Effects of stirring*

When solutions of T2 DNA containing 0·4 mg/ml. are stirred for 30 min at various speeds, no change is detectable by chromatographic comparison with unstirred DNA after stirring at speeds below 6000 rev/min. At 8000 rev/min a very considerable

change is effected, causing the stirred DNA to elute from the column at a lower salt concentration than before. Stirring at progressively higher speeds produces DNA that elutes at progressively lower salt concentrations, but always as a single band if the stirring period has not been too short. We can produce at least five chromatographically distinguishable species of DNA by stirring at various speeds up to about 25,000 rev/min, the maximum speed of our motor. In Fig. 1 we show a chromatographic comparison of three of them, representing unstirred DNA, DNA stirred at 8000 rev/min and DNA stirred at 18,000 rev/min, respectively. In interpreting the results of Fig. 1, it must be remembered that chromatographic comparison of two DNAs is essentially a test of identity or nonidentity; the distance of separation of two different DNAs along the concentration axis depends on the relative amounts of the two kinds of DNA and other factors (Mandell & Hershey, 1960).

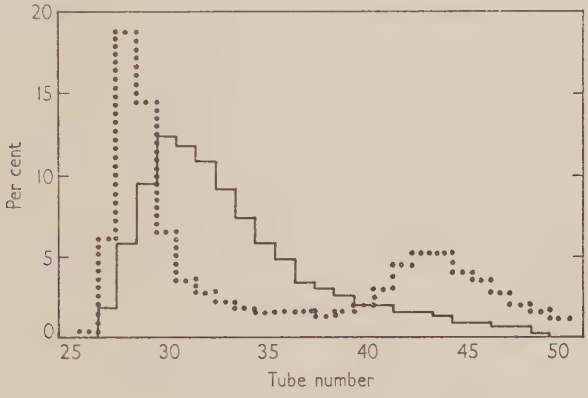

FIG. 1. Chromatographic comparison of T2 DNA stirred at 18,000 rev/min (left), 8000 rev/min (center), and unstirred (right). Solid line, D_{260} (optical density at 260 mμ); dotted line, ^{32}P.

It is important to note that no combination of speeds and times of stirring will produce DNA that elutes from the column between the positions characteristic of unstirred DNA and of DNA stirred at 8000 rev/min. This initial change is the effect of single breaks per molecule, as shown both by its all-or-none character and by the kinetics of breakage described below.

Consider first the result obtained when samples of ^{32}P-labeled DNA, diluted to contain 0·4 μg/ml. in 0·1 M-NaCl, 0·05 M-phosphate of pH 6·7, are stirred for various times at 260 rev/min, and the samples are compared chromatographically with carrier DNA that has been stirred at 0·4 mg/ml. and 8000 rev/min to produce initial breaks. Fig. 2 shows the comparison for one such sample in which 30% of the labeled DNA has been broken, and illustrates how this percentage is measured. Fig. 3 shows the kinetics of breakage, which conform to expectations if the change in chromatographic properties results from a single event per molecule. Note also in Fig. 2 that the chromatographic properties of the broken DNA in the labeled sample (30% of the total), and of the broken DNA in the unlabeled sample (60%), are the same.† Likewise, the chromatographic properties of the labeled broken DNA in the various

†Actually they are not quite the same in this instance, but for irrelevant reasons. We often find that DNA extracted from different phage preparations (not different extracts from the same preparation) are slightly different in chromatographic properties. Fig. 2 is an example of this. The same difference was seen with these two preparations of DNA when tested without stirring and when tested after stirring in mixture.

samples (always compared with the same carrier) prove to be independent of the time of stirring, at least for as long as some unbroken DNA remains. This we interpret to mean that the fragments produced by the initial breaks are not subject to further breakage at the given speed of stirring, evidently because most of the initial breaks occur near the centers of the molecules, producing fragments too short to be broken again.

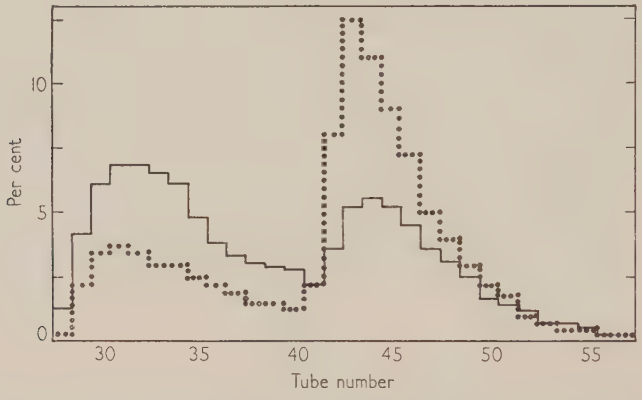

FIG. 2. Measurement of breakage. Dotted line, ^{32}P-labeled DNA stirred for 2 min at 260 rev/min and 0·4 μg/ml., 30% broken. Solid line, unlabeled DNA stirred for 15 min at 8000 rev/min and 0·4 mg/ml., 60% broken.

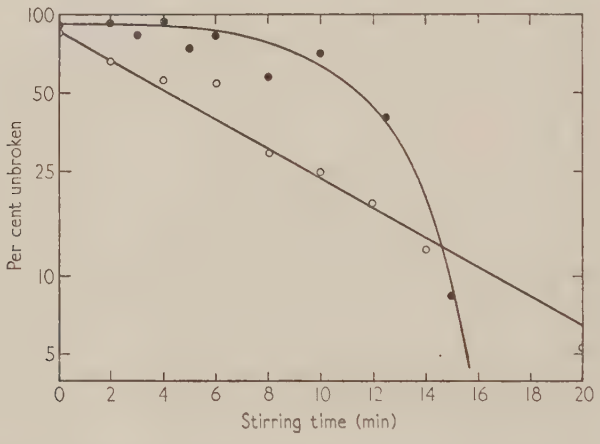

FIG. 3. Kinetics of breakage. Open circles: DNA stirred at 260 rev/min and 0·4 μg/ml. Each point is the average of 2 independent experiments. Filled circles: DNA stirred at 500 rev/min and 4 μg/ml. Measurements as in Fig. 2.

Fig. 3 also presents the contrasting result obtained when DNA is stirred at 500 rev/min and 4 μg/ml. (the same type of result is seen at 8000 rev/min and 0·4 mg/ml.). The two types of result are not inconsistent but are explained by the fact that, at appreciable concentrations, DNA exerts a strong self-protection against breakage under hydrodynamic shear, an effect that diminishes as breakage proceeds. This is proved by the experiment described in Table 1. Evidently the protective effect is sharply reduced when the molecules are broken in half, and the protective effect of unbroken DNA only disappears at some concentration between 4 and 0·4 μg/ml.

The question arises whether or not the changes in chromatographic properties produced by stirring are due solely to a reduction in molecular length. It might be supposed that the creation of unnatural ends, resulting perhaps from breaks at slightly different levels in the apposed polynucleotide chains, would also affect chromatographic behavior. If this were a predominant factor in our results, one would not expect to be able to produce a second comparable stepwise change when half-molecules are broken into quarters, because the quarters would be even more heterogeneous with respect to end-type than the halves. We therefore sought a condition under which DNA already altered by stirring could be stirred again so as to produce a mixture of two species, one of which was further altered and one not.

TABLE 1

Self-protection by DNA against breakage under shear and destruction of protective effect by stirring

Composition of mixture (μg/ml.)			
Labeled, unstirred	Unlabeled, unstirred	Unlabeled, stirred	% of labeled DNA broken
2	0	0	76, 91
2	2	0	23, 23
2	0	2	76, 82

The mixtures shown (in which the stirred DNA had been stirred for 30 min at 500 rev/min and 4 μg/ml.) were stirred for 5 min at 500 rev/min. The resulting breakage was measured as in Fig. 2. Measurements are given for two independent experiments.

This was achieved by first isolating a relatively homogeneous sample of labeled "half-molecules" by chromatographic separation of DNA that had been subjected to initial breakage, and stirring it further under carefully selected conditions. The fraction chosen for further study (8% of the total) was collected after 34% of the DNA had passed through the column, and should correspond to molecules of half the original length (Burgi & Hershey, in preparation).

When the labeled half-molecules isolated as described above were mixed with unlabeled, unfractioned DNA that had likewise been stirred to produce initial breaks, and the mixture was then stirred for 15 min at 11,000 rev/min and 0·2 mg/ml. and passed through a column, the labeled DNA separated into two bands (Fig. 4). The unlabeled DNA did not, evidently because the unfractionated half-molecules, hence *a fortiori* the quarter molecules, were diverse in length.

We also verified that labeled, fractionated, half-molecules, when stirred at 1000 rev/min and 0·4 μg/ml., survived according to an exponential function of time of stirring (18%, 47% and 73% broken in 13, 28 and 90 min, respectively). This result shows that there is no peculiarly resistant fraction with respect to the second stepwise change in chromatographic properties, in contrast to what one might expect if the column were merely registering the production of fragments with two broken ends from fragments with single broken ends.

The experiments just described also show that when half-molecules are diluted from 200 μg/ml. to 0·4 μg/ml., the speed of stirring must be reduced 11-fold to produce similar effects. In either case, the speed is critical. Half-molecules exhibit self-protection.

We conclude that the changes caused by stirring that we detect by chromatographic means are due solely or mainly to reduction in length of the molecules, which can be broken into approximate halves by initial central breaks and, at a higher speed,

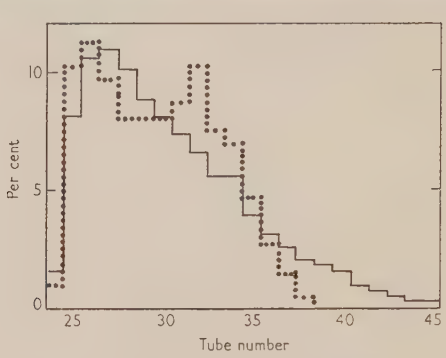

FIG. 4. Production of quarter molecules from half molecules. Dotted line, ^{32}P-labeled DNA originating from the half-molecule fraction of DNA that had been stirred for 30 min at 8000 rev/min and 0·4 mg/ml. Solid line, D_{260} of DNA previously stirred in the same way but not fractionated. The result shown was obtained after mixing the two materials and stirring the mixture again for 15 min at 11,000 rev/min and 0·2 mg/ml.

again into quarters, and so on. If this is correct, the result illustrated in Fig. 3 means that T2 DNA as we isolate it is homogeneous with respect to molecular length: otherwise progressive conversion of DNA from one length class to another by continued stirring at constant speed, without progressive alteration of either class, would be impossible. For the same reasons, it can be inferred that our DNA has not been broken during preparation by shearing phosphate ester bonds in a random manner. The possibility of breakage at specified points to produce fragments of uniform length is not, of course, excluded by our experiments.

We turn now to a more direct demonstration that our preparations of T2 DNA are chromatographically homogeneous before, and heterogeneous after, breakage by stirring.

(b) *Heterogeneity of stirred DNA*

Recall that stirring T2 DNA for 30 min at 8000 rev/min and 0·4 mg/ml. produces mainly the product of a first all-or-none change that we attribute to single breaks occurring near the centers of the molecules. If so, the product should be chromatographically heterogeneous, because the breaks could not be centered exactly. The following experiment confirms this expectation.

The starting materials were a sample of ^{32}P-labeled DNA stirred as described above, and an unlabeled sample stirred in the same manner ("stirred carrier"). The stirred, labeled DNA was submitted to a separation run through a column which yielded 15 samples of 3·6 ml. each among which the DNA was distributed in the typical manner to be illustrated below. Chromatographic comparisons showed that the labeled DNA was the same as the unlabeled DNA before stirring, and differed from it in the expected way after stirring. Additional comparisons showed that the stirred labeled DNA

before separation, was identical to the stirred carrier, and that pooled 0·1 ml. samples of the separated fractions formed a mixture showing little effect of passage through the column. The latter comparison is reproduced in Fig. 5. It suggests that only a small fraction of the slower running material in the labeled specimen had been converted into faster running material, either by breakage in the column or during transfer by pipet to reconstruct the pool. Subsequent experience confirmed the second alternative, because it was found to be exceedingly difficult to pipet dilute solutions of T2 DNA of high molecular weight without breaking it (Mandell & Hershey, 1960).

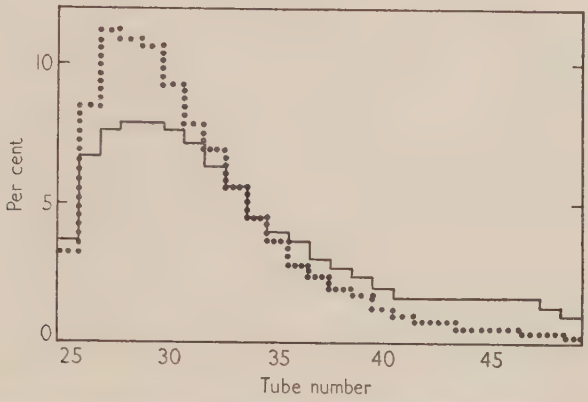

FIG. 5. Chromatographic comparison of pooled fractions from stirred DNA with the starting material. Dotted line, ^{32}P-labeled, pooled fractions from chromatographic separation of DNA stirred for 15 min at 8000 rev/min and 0·4 mg/ml. Solid line, D_{260} corresponding to unlabeled, unfractionated DNA similarly stirred.

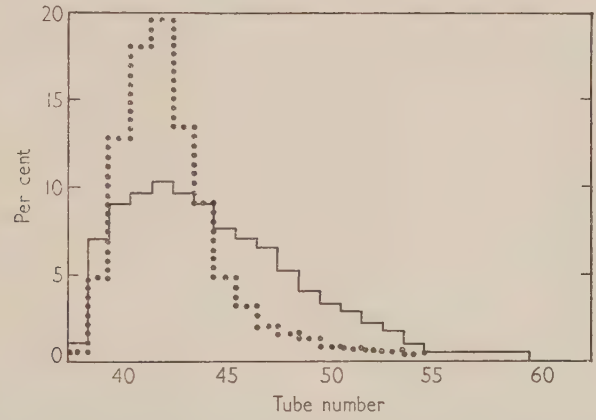

FIG. 6. Chromatographic comparison with starting material of peak fraction from stirred DNA. Dotted line, ^{32}P-labeled, peak fraction (9% of total) obtained by chromatographic separation of DNA stirred for 15 min at 8000 rev/min and 0·4 mg/ml. Solid line, D_{260}, unlabeled, unfractionated DNA similarly stirred.

Comparisons were next made by passing through the column mixtures of stirred carrier DNA, and labeled DNA taken from individual fractions obtained during the initial separation. In this way nearly all of the fractions were tested. In each case the fractionated material formed a much narrower band than the unfractionated carrier and eluted, in fact, very close to the position on the chromatogram from which it had been taken. Two examples are shown in Figs. 6 and 7.

We conclude that our column can resolve with considerable precision a heterogeneous collection of DNA molecules produced by stirring and, insofar as these may be expected to differ in molecular length, can discriminate molecules differing only slightly in length.

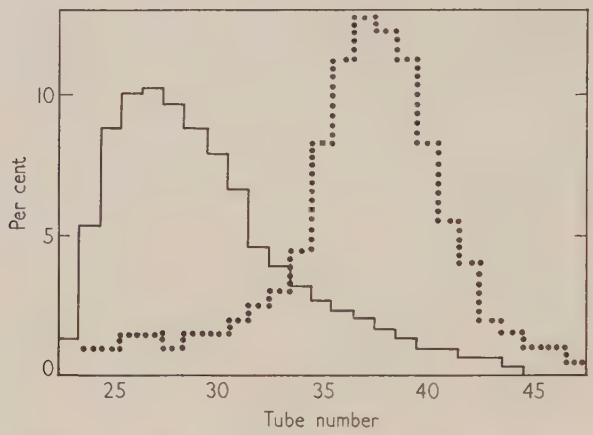

FIG. 7. Chromatographic comparison with the starting material of the 9th fraction to the right of the peak obtained with stirred DNA. Dotted line, ^{32}P-labeled DNA (3% of total) from the specified fraction obtained by chromatographic separation of DNA stirred as in Fig. 6. Solid line, D_{260}, unlabeled, unfractionated DNA stirred in the same way.

(c) *Homogeneity of native* T2 *DNA*

The following experiment is identical in other respects to the experiment just described, except that the starting material is labeled, unstirred DNA. The several fractions of labeled DNA obtained by a preliminary separation run were separately mixed with unfractionated, unlabeled carrier DNA, and the mixtures were passed

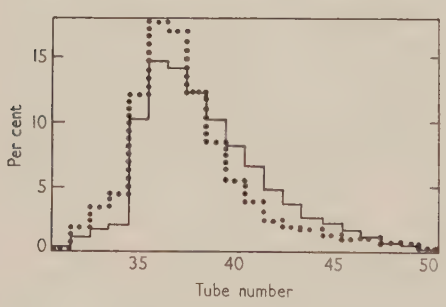

FIG. 8. Chromatographic comparison of unfractionated T2 DNA (solid line, D_{260}) with ^{32}P-labeled peak fraction (dotted line) from a previous chromatographic separation.

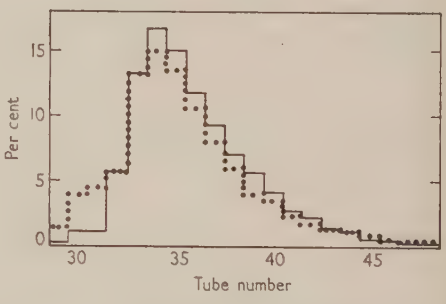

FIG 9. Chromatographic comparison of unfractionated T2 DNA (solid line D_{260}) with the 7th fraction to the right of the peak (dotted line, ^{32}P) of a previous chromatographic separation.

through additional columns. In this way nearly all of the original fractions were tested. Those comprising the small fraction of DNA found at the leading edge of the band (some 10% of the total, varying in amount in different preparations) proved indeed to differ from the remainder, resembling half-molecules. All fractions taken from the main band, including its trailing edge, however, proved to be indistinguishable from the unfractionated material. Typical results for two fractions are reproduced in Figs. 8 and 9.

4. Discussion

We find that T2 DNA subjected to stirring elutes from a column of basic protein at a lower salt concentration than does unstirred DNA. The effect is progressive and depends, when other conditions are kept constant and the time is not too short, mainly on the speed, rather than on the time, of stirring. At a minimum critical speed and after a short time of stirring, the initial change is all-or-none with respect to individual molecules. The same is true, at a higher minimum critical speed, for the transition between the product of the initial change and a second stepwise change, but only if the product of the initial change has been fractionated to render it chromatographically homogeneous. Under suitable conditions, the all-or-none character of these changes is also reflected in the survival of the original molecules according to an exponential function of time of stirring. We conclude that these stepwise changes result from transverse breaks occurring near the centers of molecular length and that the altered chromatographic behavior is due mainly or entirely to the reduction in length. If this conclusion is correct, molecules of T2 DNA can be reduced by stirring at moderate speeds to considerably less than one quarter of their original length, presumably because the original length is very great.

A point of practical importance and theoretical interest emerges from our finding that the effects of stirring at a given speed are much more severe when the DNA solution is dilute than when it is concentrated. Infinite dilution with respect to self-protection lies somewhere between 4 and 0·4 μg/ml. The protective effect (measured with respect to the production of initial breaks) is greatly reduced when the molecules are broken in half. The protection could be ascribed to a viscous effect in reducing turbulence or to a reversible molecular interaction that strengthens the molecules themselves, alternatives that we have not attempted to distinguish.

By chromatographic separation of DNA stirred to produce initial breaks, one can readily isolate DNA molecules whose mean length is apparently slightly less or slightly greater than the half-length, as identified chromatographically. By contrast, chromatographic separation of unstirred DNA does not reveal any molecules differing appreciably from the starting material, except for a very small fraction that resembles broken DNA. We conclude that DNA prepared by phenol extraction under our conditions is nearly homogeneous in molecular length and cannot have been broken in a random manner during preparation. Our main conclusion is thus at variance with evidence from radioautography (Levinthal & Thomas, 1957) that the DNA in T2 is diverse in molecular size, as well as with evidence (obtained by the use of a different column from ours) that the DNA from T2 is heterogeneous in chromatographic properties (Brown & Martin, 1955). Evidently the possibility that the earlier results were obtained with inadvertently broken preparations is not excluded. The extreme sensitivity of T2 DNA to such breakage has been pointed out before (Davison, 1959).

Our results concerning breakage by hydrodynamic shear are somewhat different from, but not inconsistent with, the results of previous work. Doty, McGill & Rice (1958) produced low molecular weight DNA by subjecting thymus DNA to sonic frequencies. They concluded that the breaks were distributed at random with respect to the original molecular lengths, yielding a very broad distribution of lengths whose mean depended on the time of treatment. We have concluded, on the contrary, that breaks caused by stirring show a strong tendency to occur near the centers of molecular length, producing a narrow distribution of lengths whose mean depends chiefly on the speed of stirring. It seems to us that these differences are readily

explained as the effects, on the one hand, of strong shearing forces acting for a short time and, on the other hand, of weak shearing forces acting for a long time. Evidently the final product obtained at any given rate of shear must consist of fragments too short to be broken by that shear, which implies at the same time that the production of fragments much shorter than the maximum length resistant to breakage is prohibited. As pointed out by Cavalieri & Rosenberg (1959), one might expect a range of lengths little greater than twofold.

Cavalieri & Rosenberg (1959) subjected pneumococcal DNA to the shearing forces produced in an atomizer operated under various pressures. They found that the resulting molecular size depended primarily on the pressure, rather than on the number of passages through the orifice. Their broken preparations were more homogeneous (with respect to sedimentation coefficient) than their starting material. Our results are the other way around, which may be due to the difference in the starting materials or to the different tests for homogeneity. Cavalieri & Rosenberg did not observe any effect of DNA concentration on sensitivity to breakage, in marked contrast to our results. Their result may be attributed to the relatively low molecular weight ($3{\cdot}5 \times 10^6$) of their starting material, or possibly to the different method of producing breaks.

The homogeneity of our preparation of DNA from T2 is to be contrasted with the heterogeneity of preparations from other sources studied in the past (Doty *et al.*, 1958). It remains to be determined whether the DNA of T2 is unique in this respect, or whether the different results merely reflect the different methods of preparation. In other respects the DNA of T4 (similar to that of T2) is unique. Doty, Marmur & Sueoka (1959) report that phage DNA, in contrast to DNA from other sources, shows an exceedingly sharp thermal denaturation temperature. According to the results of these authors, this means that the phage DNA is relatively homogeneous with respect to base composition. Presumably this method yields results that would not be affected appreciably by previous breakage of the DNA.

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The Molecular Conservation of Ribonucleic Acid during Bacterial Growth †

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The fate of ^{13}C and ^{15}N incorporated in ribosomal RNA of *E. coli* has been followed after the transfer of a uniformly labeled culture to a medium containing light isotopes alone. The distribution of heavy label among RNA molecules was determined by density-gradient centrifugation in cesium formate solution. Fully labeled molecules persisted for at least three generations while unlabeled molecules were being synthesized. No partially labeled RNA could be detected. It is concluded that no extensive exchange has taken place either with RNA precursors or with other macromolecular RNA, and that such exchange is not a characteristic feature of the synthesis or functioning of ribosomal RNA in rapidly growing *E. coli*.

1. Introduction

In connection with the problems of RNA synthesis and function, it is of interest to know if RNA molecules remain intact during the course of cellular multiplication and growth. Studies using radioactive tracers have detected no replacement of label incorporated into the RNA of exponentially growing cultures of micro-organisms (Hershey, 1954; Simonovitch & Graham, 1956). However, these experiments were unable to distinguish conservation of label on the macromolecular level from non-conservation of macromolecules due to exchange or degradation coupled with efficient salvage of the released products for further RNA synthesis.

The present experiments were undertaken in order to examine the distribution of labeled atoms on a macromolecular level. The fate of ^{13}C and ^{15}N atoms incorporated into the RNA of an exponentially growing culture of *E. coli* was followed after the transfer of a uniformly labeled culture to a medium containing only light isotopes. At intervals during subsequent growth, the distribution of heavy atoms among the population of RNA molecules was determined from equilibrium density-gradient sedimentation (Meselson, Stahl & Vinograd, 1957).

A small amount of RNA is centrifuged in a suitably concentrated solution of cesium formate. As a result of the opposing processes of sedimentation and diffusion a stable density gradient is formed in the direction of the centrifugal field. The RNA macromolecules move under the influence of the centrifugal field into the region of the

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density gradient where the solution density is equal to their own buoyant density. At equilibrium, each macromolecular species is distributed by Brownian motion over a band of width inversely related to the molecular weight. Macromolecular RNA from *E. coli* heavily labeled with ^{13}C and ^{15}N forms a band in the density gradient well resolved from that of unlabeled RNA.

2. Materials and Methods

(Additional details of the procedures described in this section are given by Davern, 1959.)

Heavy isotopes were supplied to the bacteria in the form of $^{15}NH_4Cl$ and HCl hydrolysate of $^{13}C^{15}N$-labeled *Ankistrodesmus*. The algae were grown in mineral nutrient solution in a closed system containing $^{13}CO_2$ of 67% isotopic purity and $^{15}NH_4$ of 97% isotopic purity. The algal hydrolysate was dispensed as a 10% stock solution.

Escherichia coli B uniformly labeled with ^{13}C and ^{15}N was prepared by growing cells for 8 generations to a titer of 2 to 3×10^8 in M9 medium in which nitrogen and carbon were supplied by 100 μg of $^{15}NH_4Cl$ and 0·01 ml. of 10% $^{13}C^{15}N$ algal hydrolysate per ml. The growth of the culture was followed by colony assays and direct cell counts.

An abrupt change to $^{12}C^{14}N$ medium was accomplished by the addition of 1 vol of M9 containing 2 mg/ml. of NH_4Cl, 6 mg/ml. of casamino acids, and 10 μg/ml. of each of the four ribosides; adenosine, guanosine, cytidine and uridine.

At and after the time of transfer to $^{12}C^{14}N$ medium, portions of the culture were withdrawn at intervals of approximately one generation. A volume of warm $^{12}C^{14}N$ medium equal to that of the remaining culture was added immediately after the withdrawal of each sample. In this way the bacterial concentration was kept between 1 and 3×10^8. The cells were sedimented from each portion at 1,500 ***g*** for 5 min in the cold. From this pellet lysates of whole cells, as well as lysates of purified ribosomes, were prepared for density-gradient analysis.

For the preparation of a whole cell lysate the pellet was resuspended in 0·4 ml. of a solution 0·015 M in NaCl and 0·01 M in ethylenediaminetetra-acetate (EDTA). The cells were lysed by the addition of 0·1 ml. of 10% sodium dodecyl sulfate and then frozen and stored.

For the preparation of lysates of purified ribosomes, the bacterial pellet was resuspended in 30 ml. of 0·001 M-magnesium acetate, 0·01 M-tris (2-amino-2-hydroxymethylpropane-1 : 3-diol) at pH 7·4 and again sedimented. The pellet was resuspended in 5 ml. of the tris magnesium acetate buffer, containing 2·5 μg of DNase per ml. This suspension was shaken in the cold for 2 min with 5 g of Ballotini glass beads (grade 10) in a Nossal shaker (Nossal, 1953). The disrupted cell suspension and 4 ml. of washings from the glass beads were centrifuged for 15 min at 3,500 ***g*** and the supernatant was frozen for storage. The thawed supernatant was made up to 11 ml. with buffer and was centrifuged in a Spinco No. 40 rotor for 6 hr at 40,000 rev/min. The ribosomes were lysed by dispersing the pellet in 0·5 ml. of a 2% solution of sodium dodecyl sulfate 0·015 M in NaCl and 0·01 M in EDTA and then frozen until needed for density-gradient centrifugation.

High purity cesium chloride was obtained from the Maywood Chemical Works, Maywood, New Jersey. Cesium formate was prepared from cesium chloride by anion exchange in Dowex 2-X8, 200–400 mesh anion exchange resin in the formate form. The resulting cesium formate solution was treated with acid washed decolorizing charcoal to remove u.v.-absorbing material. After filtration, it was brought to pH 8·5 with concentrated formic acid and evaporated under vacuum to give a stock solution with density 2·32 g/cm^{-3} and optical density 0·12 at 254 mμ.

Thawed whole cell or ribosomal lysate was added to an aliquot of cesium formate stock solution and the density was adjusted to approximately 2·08 g/cm^{-3} by the addition of water. The density at 20°C, ρ^{20}, was calculated from the refractive index $n_D{}^{25}$ for sodium light at 25°C according to the following relationship:

$$\rho^{20} = 12{\cdot}876 n_D^{25} - 16{\cdot}209 \text{ g/cm}^{-3} \quad (1)$$

For each centrifugation, 0·7 ml. of the cesium formate lysate solution was placed in a 12 mm, 4° centrifuge cell fitted with a Kel-F centerpiece and a −1° prismatic window.

Solutions were centrifuged at 52,640 rev/min at 20°C in a Spinco model E ultracentrifuge for at least 53 hr.

The density gradient approaches closely to equilibrium in approximately 10 hr. Its value is then 0·087 g/cm^{-4} as calculated from equation (1) and the gradient of refractive index observed in the schlieren optical system of the ultracentrifuge. This calculation ignores the additional density gradient caused by compression, which would be 0·03 gm/cm^{-4} if the cesium formate solution were as compressible as water.

Portions of the bacterial lysates were analyzed also for the density distribution of DNA in a CsCl density gradient. Samples were prepared by the procedure employed by Meselson & Stahl (1958) and were centrifuged at 44,770 rev/min at 25°C for at least 25 hr.

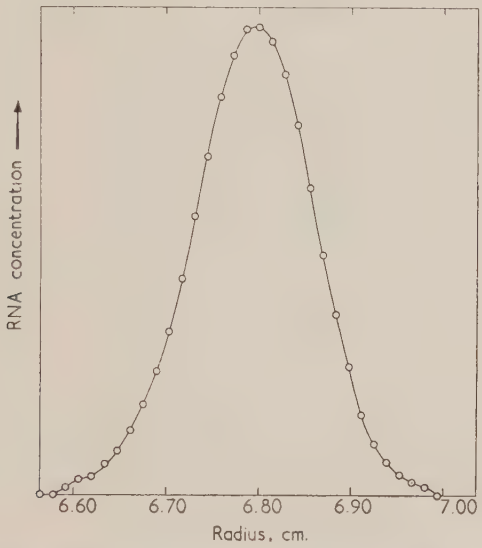

Fig. 1. Microdensitometer tracing showing the concentration distribution of RNA from a whole cell lysate after 80 hr of centrifugation at 52,640 rev/min at 20°C.

Ultraviolet absorption photographs recording the distribution of RNA or DNA in the centrifuge cell were taken during the course of each centrifugation and were scanned with a double beam automatic recording microdensitometer.

At equilibrium, the concentration distribution of RNA from purified ribosomes closely resembles the distribution of RNA from whole cell lysates. Both distributions are very nearly Gaussian (Figs. 1 and 2) but that of whole cell RNA exhibits a slight shoulder on either side of the band. This feature may be due to soluble RNA reported to have a molecular weight of 25,500 (Tissières, 1959). In the absence of information regarding density heterogeneity, density-gradient centrifugation can establish only a lower bound on molecular weight (Meselson *et al.*, 1957). For the RNA examined in these experiments the minimum weight-average molecular weight, obtained from the standard deviation of the distribution shown in Fig. 1, is 450,000. This value refers to the sodium salt and was calculated neglecting the contribution of compressibility to the density gradient. It may be compared with the value 555,000 given by Tissières, Watson, Schlessinger & Hollingworth (1959) for the individual RNA content of 30 s ribosomes from *E. coli*.

During density-gradient centrifugation of either whole cell or ribosomal ysates, the first separation of $^{13}C^{15}N$ RNA and $^{12}C^{14}N$ RNA into discrete bands occurs after about 22 hr. Thereafter the means of the two bands recede from one another at a rate which decreases exponentially with time. Determinations of the RNA distribution in

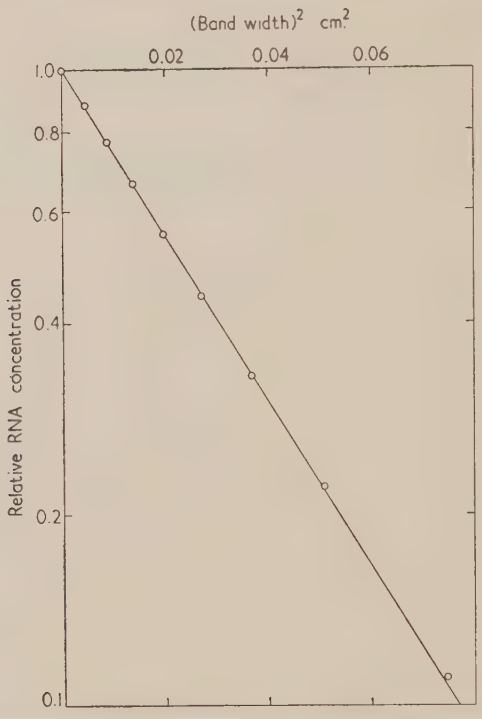

FIG. 2. A plot of the log of the RNA concentration shown in Fig. 1 against the square of the band width.

the density gradient for the various samples were made from microdensitometer tracings of u.v. absorption photographs taken between the 53rd and 61st hour of centrifugation. Over this 8 hr period the separation between the bands increased by 2%. Extrapolation based on the exponential character described above indicates the equilibrium separation to be only 4% greater than that observed at 61 hr.

3. Transfer Experiments

Two transfer experiments were performed with essentially similar results. In the first the whole cell lysates were examined directly in the density gradient; in the second, density gradient analysis was performed on lysates of purified ribosomal particles. A record of the growth of the bacterial populations is shown in Fig. 3.

Experiment 1

Ultraviolet absorption photographs of the final RNA distributions for the zero-time sample, the 4 post-transfer samples, and a sample containing a mixture of the zero-time and fourth post-transfer samples are shown in Plate I. Microdensitometer tracings of these photographs are presented in Fig. 4. For comparison, the results of the density-gradient centrifugation of DNA from the first 4 samples are shown in the

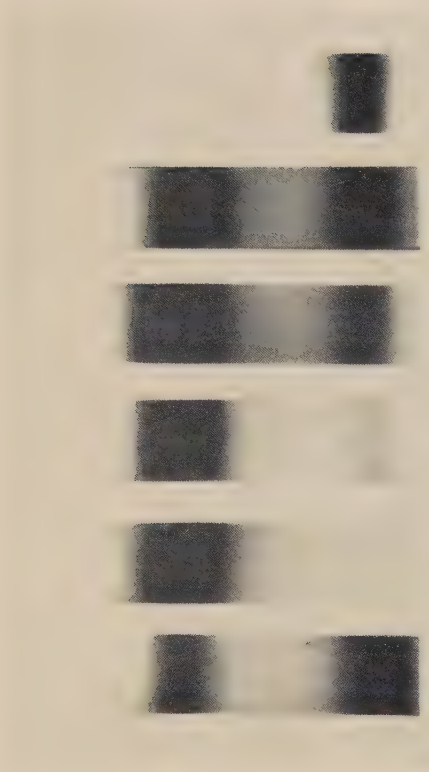

PLATE I. Ultraviolet absorption photographs showing RNA bands resulting from density-gradient centrifugation of lysates of bacteria sampled at various times after transfer from heavy to light medium. The photographs correspond, in descending order, to samples taken at 0, 33, 66, 100, and 133 min after transfer. The bottom photograph shows a mixture of the 0 and 133 min samples. Density increases to the right.

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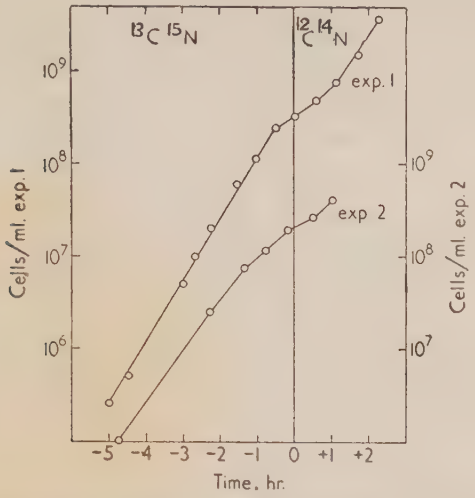

FIG. 3. Growth of the bacterial populations of experiments 1 and 2. The values on the ordinates are the actual titers of the cultures up to the time of addition of $^{12}C^{14}N$. Thereafter, the actual titer was kept between 1 and 3×10^8 by additions of fresh medium. The titers shown during the later period are corrected for these dilutions.

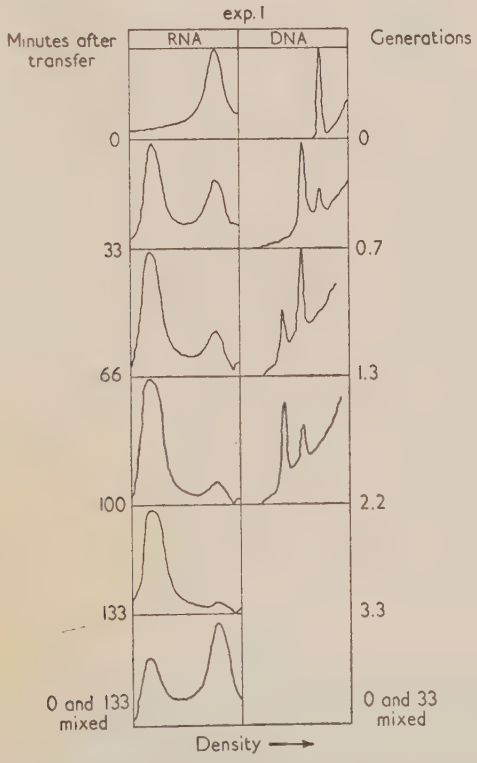

FIG. 4. Microdensitometer tracings of u.v. absorption photographs from experiment 1, showing ribosomal RNA bands (left) and DNA bands (right) resulting from density-gradient centrifugation in cesium formate and cesium chloride, respectively, of lysates of bacteria sampled at various times after the addition of an excess of $^{12}C^{14}N$ substrates to a growing $^{13}C^{15}N$-labeled culture. Regions of equal density are within 3% of the same horizontal position in each tracing. The vertical displacement of the tracing is only approximately proportional to the concentration of RNA and DNA respectively. The generation times were estimated from the measurements of bacterial growth presented in Fig. 3.

O

same figure. It may be seen that the RNA from each of the post-transfer samples is confined to two discrete bands, one corresponding to $^{13}C^{15}N$ RNA and the other to $^{12}C^{14}N$ RNA.

A comparison of the microdensitometer record from the 33 min sample with that from the combined 0 and 133 min samples gives no evidence for the existence of partially labeled RNA.

TABLE 1

Distance separating modes of RNA bands in the density gradient at equilibrium

Experiment No.	Sample (time after transfer, min)	Time centrifuged at 52,640 rev/min (hr)	Distance between band modes (cm)	Distance corrected for centrifugation time and gradient (cm)
1	33	53	0·54	0·57
	66	58	0·56	0·59
	99	54	0·54	0·57
	133	58	0·56	0·59
	0+133	61	0·59	0·59
2	30	60	0·53	0·55
	60	60	0·56	0·58

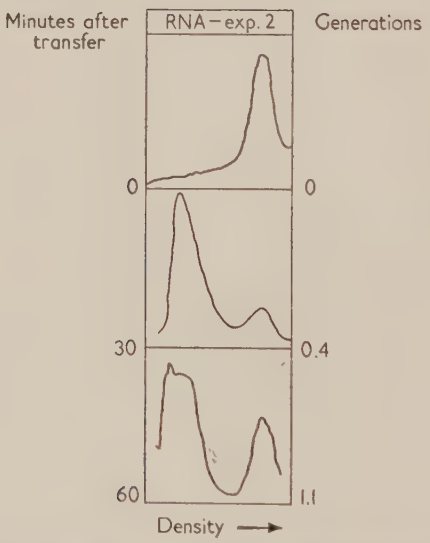

FIG. 5. Microdensitometer tracings of u.v. absorption photographs from experiment 2 showing ribosomal RNA bands resulting from density-gradient centrifugation in cesium formate solution of lysates of purified ribosomes prepared from bacteria sampled at various times after the addition of an excess of $^{12}C^{14}N$ substrates to a growing $^{13}C^{15}N$-labeled culture.

The distance between the modes of the RNA bands in each post-transfer sample is given in Table 1. These distances were measured after subtraction of an interpolated base line and include slight corrections for variation among the samples in the value

of the density gradient in the region of RNA-band formation (the value of the density gradient varies directly with distance from the axis of rotation). The distances are normalized to a common centrifugation time of 60 hr.

Experiment 2

The results of experiment 2 closely resembled those of experiment 1. The microdensitometer tracings of the RNA distributions in the density gradients for the zero-time and two post-transfer samples are shown in Fig. 5. The distances between the modes of the two RNA bands appearing in the two post-transfer samples are listed in Table 1.

Within the accuracy of measurement, it may be seen from the data of Table 1 that the density difference between the modes of heavy and light RNA remained constant throughout both experiments.

4. Discussion

The density distribution of DNA, determined for the first four samples of experiment 1, is in complete agreement with that found in an earlier experiment using ^{15}N label alone (Meselson & Stahl, 1958). Following the interpretation given for that experiment, it may be concluded that the carbon and the nitrogen of the DNA molecules of *E. coli* are divided equally between two subunits. The subunits dissociate at each generation and become associated with newly synthesized subunits.

The relevance of the DNA results to the present experiment concerns the possibility that fully labeled RNA molecules found in post-transfer samples have merely come from synthetically inactive cells. The complete disappearance of fully labeled DNA molecules after the passage of one generation provides evidence that essentially all the cells are synthetically active.

The predominance of light over heavy RNA apparent in Figs. 4 and 5 even before one generation has passed indicates that the cells were not in a steady state of growth but that, instead, RNA synthesis ran ahead of both cellular division and DNA synthesis during the adaptation of the cells to the new medium. A less likely hypothesis, which cannot however be excluded in these experiments, is that heavy RNA is extensively degraded and *not* re-incorporated into macromolecular RNA. Such loss of RNA does not occur in exponentially growing cultures of *E. coli* (Hershey, 1954). The possibility also exists that label initially observed in heavy RNA passes into a special and abundant RNA fraction which does not appear in the density region examined. This possibility is made unlikely by the observation that the amount of RNA found in the region of density 2·0 g/cm^{-3} was approximately that expected on the basis of the total RNA content of *E. coli*.

Both the absence of detectable amounts of partially labeled material in the region between the modes of heavy and light RNA and the constancy of the inter-modal density difference demonstrate that the precursors from which any given RNA molecule was ultimately synthesized were drawn from the medium during an interval which was short relative to the generation time of the culture. It may also be concluded that RNA molecules did not participate in extensive exchange reactions either with precursors, or among themselves, unless such exchange occurred only between molecules synthesized within a very short time of one another.

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Letters to the Editor

Some Properties of DNA from Shope Papilloma Virus

Few biological sources yield DNA molecules all of which are identical. Generally cells contain many genetically distinct DNA molecules and there is as yet no way to separate them. The very small bacteriophage ϕX174 and its relative S13 are the only sources known so far to contain but one DNA molecule; and their DNA is unique in that it is single stranded (Sinsheimer, 1959). The Shope papilloma virus might also be a source of homogeneous DNA. Its molecular weight is reported to be 47×10^6 (Neurath *et al.*, 1941), with a DNA content of about 8% (Taylor, Beard, Sharp & Beard, 1942). If these measurements refer to infectious virus particles, the DNA complement per particle would be about 4 million, which is less than most reported values for double-helical DNA molecules (Butler, Laurence, Robins & Shooter, 1959). Here we report some preliminary results on DNA isolated from preparations of Shope papilloma virus.

Viral Purification and DNA Isolation

Papillomas from cottontail rabbits were obtained during the winter 1958 to 1959 by Earl Johnson, Rago, Kansas, and stored at 4°C in a solution containing equal parts of glycerin and phosphate-buffered saline (0·137 M-NaCl, 0·0027 M-KCl, 0·008 M-Na_2HPO_4 and 0·0015 M-KH_2PO_4). The virus was prepared essentially by the method of Beard, Bryan & Wyckoff (1939) as modified by Knight (1950). In our two preparations the papillomas, freed of connective tissue, were disrupted in a Waring blendor and purified by three cycles of differential centrifugation (each cycle 7000 ***g*** for 15 min followed by 80,000 ***g*** for 1 hr). The pellets were suspended in buffered saline containing 0·001 M-$MgCl_2$ adjusted to pH 7. 18·6 and 25·6 mg of virus protein were obtained from 132 and 130 gm of papillomas. This was an average of 0·16 mg per gm of wet papillomas which, from the results of Beard *et al.* (1939), constitutes a moderate yield. In both cases dilutions up to approximately 10^{-9} gm of virus produced a papilloma when placed on the lightly scarified skin of domestic rabbits. This indicates an infectivity again comparable to that reported by Beard *et al.* (1939).

The DNA contents of our two preparations were 6·6 and 7·1% (methods of Kissane & Robins (1958) for DNA; of Lowry, Rosebrough, Farr, & Randall (1951) for protein; and of Ma & Zuazaga (1942) for total N). No RNA was found by methods which would have detected 0·2% RNA (no uracil; no ultraviolet absorbing material released by alkaline digestion).

Both preparations of virus were examined in the Spinco model E analytical centrifuge using schlieren optics. Three components were present in each preparation. The main component, which accounted for about 65% of the material, had a sedimentation coefficient of 290 s. The remaining material was almost equally divided between 180 s and 410 s components. These figures are similar to those reported by Schachman (1951).

DNA was isolated from the virus by the use of Duponol C (sodium lauryl sulfate) (Marko & Butler, 1951). 0·25 vol of 5% Duponol in ethanol : water (45 : 55) was added to a virus suspension containing about 1 mg of virus per ml. of buffered saline. At

room temperature the virus did not break down, as indicated by persistence of the blue opalescence. A few minutes at 50°C, however, were sufficient to cause complete clearing. After 30 min at 50°C, 0·25 vol of 5 M-NaCl was added to cause precipitation of virus protein and excess Duponol. After 10 hr at 4°C, the precipitate was removed by centrifuging at 15,000 *g* for 30 min. 72 to 74% of DNA remained in the supernatant while most of the viral protein was removed; the ratio of optical densities at 260 mμ/230 mμ was 1·95. The DNA was then twice precipitated by the addition of ethanol and redissolved in buffered saline. One sample was purified further by centrifugation in a CsCl solution of density 1·45 for 10 hr at 35,000 rev/min in the SW 39 Spinco swinging bucket rotor. The residual protein floated to the top while the DNA formed a tiny translucent pellet at the bottom of the tube. This pellet, resuspended in buffered saline, was effectively protein-free, as judged from its high 260 mμ/230 mμ ratio of 2·7. Though this sample was more pure, its physical measurements (see below) were indistinguishable from the protein-contaminated samples.

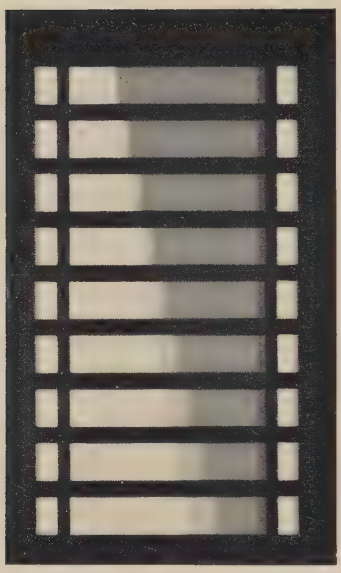
Base Composition

Three methods were used to determine base composition.

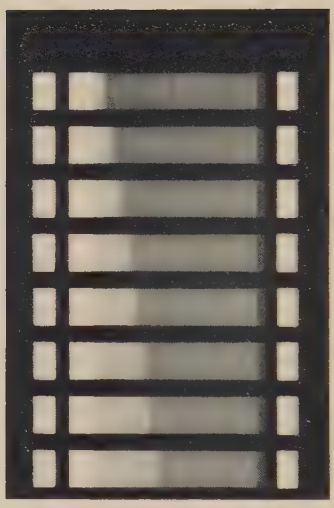
(1) One purified DNA sample was hydrolysed with concentrated perchloric acid (72% w/v) at 100°C for 1 hr; then the bases were separated by two-dimensional chromatography using *iso*propanol : H_2O : concentrated HCl (657 : 176 : 167) followed by *n*-butanol : H_2O (86 : 14) with NH_3 in the vapor phase, located by ultraviolet photography, eluted in 0·1 N-HCl, and estimated spectrophotometrically against appropriate blanks (Wyatt, 1955). This analysis gave molar ratios of adenine 1·00, thymine 1·03, guanine 0·94, and cytosine 0·89. Since the recovery of bases accounted for only 72% of the DNA extracted with Duponol, and the latter represented but 72% of the viral DNA, the analysis was repeated directly on a portion of whole virus from the second preparation (Smith & Wyatt, 1951). Virus containing 440 μg of DNA was precipitated with 5% trichloroacetic acid at 4°C, and washed twice with cold trichloroacetic acid, once with 0·1 M-alcoholic potassium acetate, and twice with ethanol. The base composition was then determined as before. 97% of the virus phosphorus (Lowry, Roberts, Leiner, Wu & Farr, 1954) was present in the hydrolysate, and the recovered bases accounted for 98% of this value. The molar ratios found were adenine (A) 1·00, thymine (T) 0·93, guanine (G) 0·92, and cytosine (C) 0·91. The average ratio of A+T/G+C for these determinations was 1·08.

(2) Since Shope papilloma virus DNA is double helical (see below), the A+T/G+C ratio can be measured in two additional ways. One involves the temperature (T_m) at which half the DNA molecules are denatured. Marmur & Doty (1959) have shown that T_m is directly proportional to the A+T/G+C ratio. T_m is easily measured by observing the ultraviolet absorption at 260 mμ as a function of temperature. When DNA denatures, 260 mμ absorption increases (decrease in hypochromicity). Virtually no absorption change was observed for papilloma DNA (suspended in 0·15 M-NaCl and 0·01 M-Na citrate pH 6·8) until about 85°C when a slight increase occurred followed by a rapid increase between 87°C and 92°C. At higher temperatures only a slight additional increase occurred, the total increase being about 45% of the value at 25°C. This sharp melting behavior (Marmur & Doty, 1959) is characteristic of double helical DNA and the observed T_m of 89·5°C indicates an A+T/G+C ratio of 1·04.

(3) Another measure of the A+T/G+C ratio comes from its density in CsCl solutions. Sueoka, Marmur & Doty (1959) have shown that DNA density is directly pro-



(a)



(b)

PLATE I (a) Ultracentrifuge sedimentation pattern of freshly prepared papilloma DNA (13 μg/ml.) in 0·01 M-tris buffer pH 7·4 and 0·15 M-NaCl at 21·5°C. Pictures taken every 4 min at 42,040 rev/min.

(b) Ultracentrifuge sedimentation pattern of aged papilloma DNA (11 μg/ml.) in 0·1 M-tris buffer pH 7·4 and 0·15 M-NaCl at 19·5°C. Pictures taken every 4 min at 42,040 rev/min.

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portional to the A+T/G+C ratio. Our experiments (see below) in which we measured the molecular weight of papilloma DNA using the density gradient equilibrium method of Meselson, Stahl & Vinograd (1957) yielded a density in CsCl of 1·714 (a ^{15}N-labelled *Serratia* DNA preparation calibrated against *E. coli* DNA of density 1·713 was mixed with the papilloma DNA to serve as a reference density marker). This corresponds to an A+T/G+C ratio of 1·02.

Sedimentation Analysis

Three papilloma DNA preparations have been examined in the Spinco model E centrifuge using ultraviolet optics, the B rotor, and DNA concentrations ranging from 7 to 15 μg/ml. All preparations (in 0·01 M-tris (2-amino-2-hydroxymethylpropane-1 : 3-diol) buffer and 0·15 M-NaCl) originally contained two very homogeneous components of 28·5 s and 21 s (Plate I(a)). Both components appear equally homogeneous, with the slower moving component amounting to about 65% of the total ultraviolet absorbing material. Continued incubation at 4°C of the DNA frequently results in the conversion of the faster component to the slower component (Plate 1(b)). Attempts to hasten this conversion through changes in salt concentration, pH, or addition of chelating agents, have not been successful. Moreover, heating one sample briefly to 95°C and recooling to room temperature left both components intact with no change in their relative amounts. In our several experiments, the heated DNA samples upon recooling appeared to renature (see Doty, Marmur, Eigner & Schildkraut, 1960) almost completely, as judged by the existence of sharp melting behavior (T_m=86 to 88°C) when these samples are reheated a second (or third or fourth) time.

Use of the empirical relationships of Doty, McGill & Rice (1958) suggests molecular weights of 7 and 14 million for the 21 s and 28 s components. Since there was insufficient Shope papilloma virus DNA for light scattering measurements, the CsCl density gradient equilibrium method (Meselson *et al.*, 1957) was used to measure the molecular weight. The very sharp melting behavior indicates very little density heterogeneity in this DNA; hence the band shape should only be a function of molecular weight. Two DNA samples, each containing several μg of exclusively 21 s material, were centrifuged in CsCl of density 1·72 for 65 hr at 35,400 rev/min. Ultraviolet photographs of varying exposure time were taken and photometered with a Joyce-Loebl double beam recording densitometer. The band shape was then used to calculate a molecular weight. The two runs, one employing 3 μg and the other 2 μg of DNA, gave molecular weights of 4·9 and 5·3 million. The close agreement between these values and the 7 million value from the formula of Doty *et al.* (1958) suggests that Shope papilloma virus DNA is physically similar to other double helical DNA.

Number of DNA Molecules per Virus Particle

The principal component in Shope papilloma virus preparations has a sedimentation coefficient of 285 s, a molecular weight about 47 million, and is generally believed to be the infectious particle (Neurath *et al.*, 1941). The DNA content of our preparations, like those reported earlier by Taylor *et al.* (1942), are between 6 and 8 %. Thus, if all the preparation was infectious virus, the maximum DNA content per virus particle would be about 4 million. However, the DNA molecules of 21 s probably have a molecular weight near 7 million and the 28 s material is likely to be 14 million. Two questions thus arise: (1) what is the proportion of complete virus particles in our preparations, and (2) does the amount of DNA per complete infectious particle correspond to the 21 s or 28 s DNA component?

Several facts indicate that Shope papilloma virus preparations are not homogeneous but contain many incomplete particles. First, sedimentation diagrams reveal a slower moving component (185 s) in addition to the 285 s particle. This material, judged from its area in the schlieren diagram, may account for 25% of the total preparation. There is also a faster component of about 400 s, which may be a dimer of the 285 s material, but if not, adds another 15 to 20% of non-infectious material. Incomplete particles are also suggested by electron micrographs taken by Dr. Hugh Huxley (personal communication), employing negative staining techniques (Huxley, 1956). These photographs reveal that approximately 25% of the particles are hollow. Thus the nucleic acid content of complete particles must be higher than 8%, and is probably 12% or greater.

The 28 s DNA molecule seems likely to have just twice the molecular weight of the 21 s molecule. This is hardly chance and suggests that infectious Shope papilloma virus particles may contain two 21 s DNA molecules held together by an unstable connecting link. If this is so, the DNA complement of the infectious particle would be 14 million, which would correspond to approximately 30% by weight of a 47 million molecular weight virus. It is probable that the presence of incomplete particles resulted in too low a value for the molecular weight as reported by Neurath *et al.* (1941), but it seems unlikely that the molecular weight of the 285 s particle is greater than 60 million. Alternatively, the 28 s DNA molecule may arise from end-to-end dimerization of 21 s DNA *after* extraction of the DNA from the virus particles. If so, this would be the first known case of stable DNA dimers.

Future experiments must utilize homogeneous virus preparations. As the virus appears to be stable in CsCl, density gradient purification of complete infectious particles should be possible. Our experiments have been interrupted by lack of virus, but when more becomes available further work along these lines is planned.

We are indebted to Dr. N. Sueoka for assistance with the cesium chloride experiments and calculations. This investigation was supported in part by Research Grant C-2146 from the National Cancer Institute (USPHS) and an Institutional Grant from the American Cancer Society to the Massachusetts General Hospital.

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Properties of Low Molecular Weight RNA from *E. Coli* †

High molecular weight *E. coli* RNA (H-RNA) has been shown to behave in solution as a flexible single-stranded molecule (Eisenberg & Littauer, 1958; Littauer & Eisenberg, 1959) possessing an organized secondary structure (Cox & Littauer, 1959; 1960, in preparation). Secondary organized structure has also been reported for tobacco mosaic virus RNA (Geirer, 1957; Boedtker, 1959; Haschemeyer, Singer & Fraenkel-Conrat, 1959; Doty, Boedtker, Fresco, Haselkorn & Litt, 1959; Spirin, Gavrilova, Bresler & Mossevitsky, 1959) and calf liver microsomal RNA (Doty *et al.*, 1959; Hall & Doty, 1959).

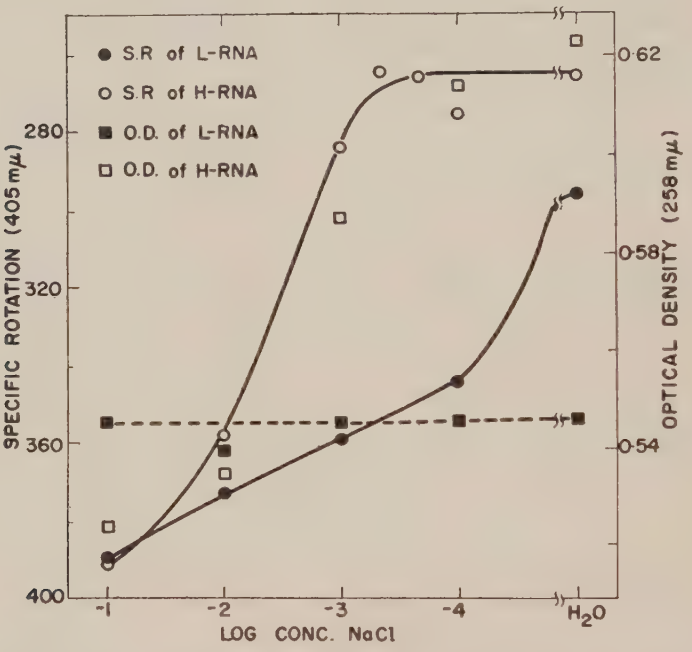

FIG. 1. The variation of the specific rotation and optical density of L-RNA and H-RNA at 25°C as a function of NaCl concentration.

The results presented below indicate that there are differences in the behavior of *E. coli* low molecular weight RNA (L-RNA) (which is probably homologous to the "soluble" RNA of higher organisms) and H-RNA, in solution. The L-RNA was prepared as previously reported (Littauer & Eisenberg, 1958; 1959). It revealed a single broad peak in the ultracentrifuge ($S_{20} = 4{\cdot}1$ in 0·2 M-NaCl containing 0·05 M-phosphate buffer pH 6·8). The viscosity numbers (η_{sp}/c) of L-RNA (at a concentration of 1·37 mg/ml.) showed only a small dependence on the ionic strength of the solution. The viscosity numbers in H_2O, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M-NaCl were 34, 33, 24, 15 and 14 ml./g respectively. The total change from very low values of NaCl concentration to the higher values corresponded to about a twofold decrease in

†This research was supported in part by a United States Public Health Service research grant R.G.-5217.

viscosity, in contrast to a 100-fold decrease observed for H-RNA. The molecular weight of L-RNA computed from the Scheraga-Mandelkern equation is about 27,000 to 30,000.

Evidence for a secondary structure involving helical regions was obtained from optical rotation measurements. These were performed at various concentrations of NaCl. The values of the specific rotation, $[\alpha]_{405}$, of both preparations were almost identical in 0·1 M-NaCl solution (Fig. 1). The specific rotation of H-RNA decreased by about one-third when the salt concentration was reduced to 1×10^{-3} M, whereas a similar sharp decrease was found for L-RNA only in solutions more dilute than 1×10^{-4} M-sodium chloride. Likewise the optical density of L-RNA was little affected by ionic strength, whereas that of H-RNA changed substantially, and paralleled the decrease in the specific rotation values. The effects measured were found to be reversible. The molar extinction coefficient with respect to phosphorus, ϵ(P), at 260 mμ in 0·1 M-NaCl for L-RNA was 7,880 compared with 7,290 for H-RNA.

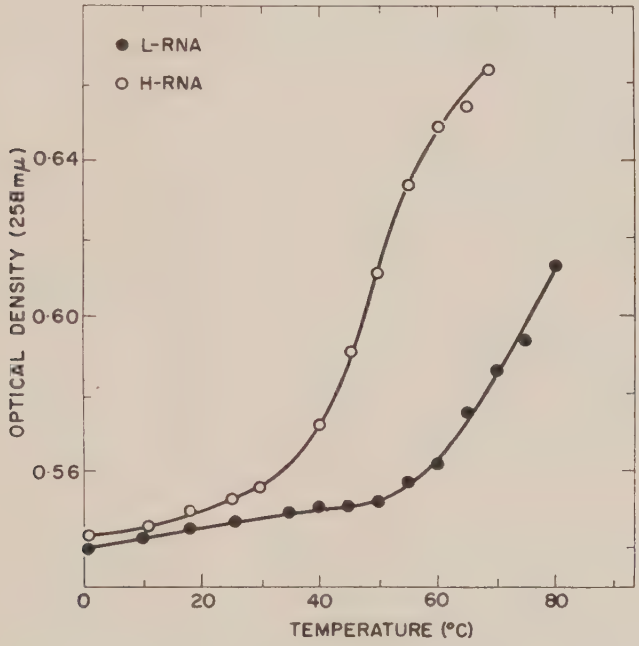

Fig. 2. Effect of temperature on optical density of L-RNA (filled circles) and H-RNA (open circles) in 0·1 M-NaCl.

The optical density at 258 mμ of both L-RNA and H-RNA was followed as a function of temperature (see Fig. 2). In both cases changes were found which are believed to accompany the transition from helix to coil (Doty *et al.* 1959), although the curve for L-RNA was displaced in a parallel fashion by about 26° at higher temperatures. This is an indication that the helices of L-RNA are more stable to temperature, as well as to ionic strength, than those of H-RNA.

The stability of helices are believed to depend on the number of base pairs per helical region, the number of imperfections, and the relative abundance of the base pairs guanine:cytosine and adenine:uracil, since the former pair form the stronger hydrogen-bonds (Marmur & Doty, 1959). Thus the increased stability of L-RNA might be accounted for by the presence of a greater number of guanine:cytosine

hydrogen bonded base pairs. This is indicated by the near equivalence of these bases (32·1 moles % guanine; 28·9 moles % cytosine) (Spahr, private communication), which is not found in H-RNA (guanine 28·9 moles %; cytosine 22·0 moles %) (Littauer & Eisenberg, 1959). The presence of unusual bases (Dunn, 1959) is less likely to contribute to the observed differences in stability. The value of the specific rotation ($[\alpha]_D = 134$) in 0·1 M-NaCl indicates that about 45% of the bases might have a helical environment (Doty *et al.*, 1959).

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Three Amino Acids at the Active Site of Beta Amylase †

The number of amino acids necessary for enzyme action is of importance to theories of enzyme catalysis and to the elucidation of the genetic determinants of protein structure. In this communication we wish to report studies on β-amylase leading to the identification of amino acids at its active site.

Twice crystallized β-amylase was incubated with 0·1 M-iodoacetamide in 0·05 M-pyrophosphate buffer (pH 8·8) at 25°C. At various times portions were withdrawn, diluted 100-fold to stop the alkylation reaction, and the enzyme assayed for hydrolytic activity. The rate of enzyme destruction followed pseudo first order kinetics to about 90% inactivation and then leveled off to give, after 5 hr, a modified enzyme

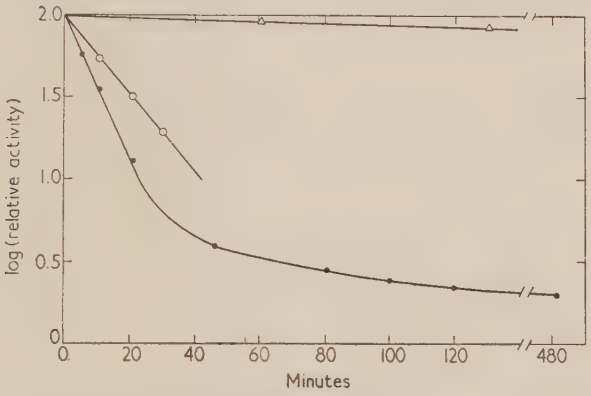

FIG. 1. Effect of time on the inactivation of β-amylase by iodoacetamide. ○, in presence of cyclohexaamylose; ●, in absence of cyclohexaamylose; △, in absence of iodoacetamide.

with 2% of the initial activity (see Fig. 1). The affinity of cyclohexaamylose for the modified enzyme decreased by a factor of 8. This fact is consistent with the work of Rowe & Weill (1958) who showed that *p*-chloromercuribenzoate is a competitive inhibitor of β-amylase. Titrations by the method of Boyer (1954) and amino acid analyses by the method of Spackman, Stein & Moore (1958) showed that all the reactive sulfhydryl groups and only the sulfhydryl groups were alkylated in this modified enzyme. Furthermore, it was found that cyclohexaamylose, a competitive inhibitor of the enzyme, protected against iodoacetamide inactivation (*cf.* Fig. 1). These data indicate that a sulfhydryl group is at the active site and that carboxymethylation decreases the catalytic power by a factor of 50 and the binding affinity by a factor of 8.

To identify other groups involved in the catalytic action, a pH-V_m study was made on both the native and the modified enzyme (*cf.* Fig. 2). The group with pK 3·7 is undoubtedly a carboxyl group, while the group with pK 7·0 in the native enzyme could be an α-amino, sulfhydryl, or imidazole group. α-Amino can probably be eliminated as the pK 7 group because of the acylation studies of Weill & Caldwell (1945),

† Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

and a sulfhydryl group can be excluded because of the similarity of the pH dependence of the native and modified enzyme (*cf.* Fig. 2). Consequently, imidazole in its protonated form is implicated in the catalysis. Further support for the necessity of imidazole was obtained by photoöxidation of the enzyme with methylene blue because the enzyme could be inactivated until only 0·1% of the initial activity remained. Since a $-\log K_m$ *vs* pH plot showed only a slight pH variation, these data in conjunction with the large pH dependence of V_m suggest that the primary role of carboxyl and histidine is to catalyze the hydrolysis.

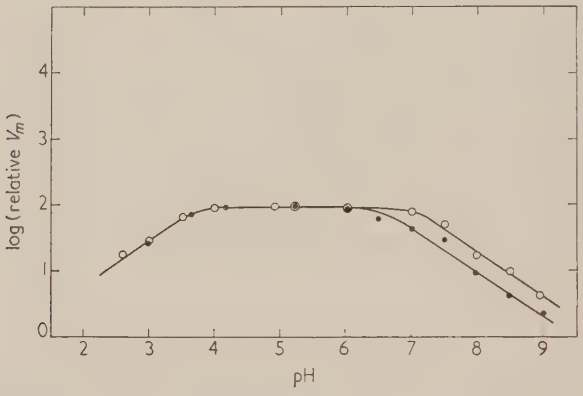

FIG. 2. pH-activity profile for native and iodoacetamide-inactivated β-amylase. ⊙, native; ●, alkylated.

It can be concluded that a carboxyl group, an imidazole group, and at least one sulfhydryl group are at the active site and are involved in the enzyme action of β-amylase.

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